A methodical approach for non-destructive estimation of plant pigments by means of remission spectroscopy applied in fruit and vegetable analyses

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Abstract

The biological variability of plant material often leads to perturbations when optical recordings are carried out non-destructively on living horticultural products or even within the photometric analysis of plant extracts. Perturbations are related to varying and coinciding absorption and scattering coefficients. Changing spectral properties appear due to different cultivars, and growing season and also occurs during the development of fruit. An instrumental analysis of varying optical attributes consequently requires accurate methodological specifications. To date, spectral measurements have already been introduced in practice through extensive research in the field of plant spectroscopy and through the recent increase in the availability of low-cost devices. It can be expected that the use of optical sensor systems may contribute to an economic and sustainable use of natural resources as a part of the concept for precision horticulture. In terms of optical phytomonitoring approaches, technologies which address variable amounts of different and thus individual chromophoric plant components, better known as plant pigments, become important. Their wavelength-selective light absorption makes pigments specifically responsive to reflection or transmission recordings in the ultraviolet and visible (UV/VIS) range of the electromagnetic spectrum. Along with their chromophoric attributes, pigments serve as indicators for physiological stages of leaf and fruit. Consequently, the instrumental acquisition of changing pigment contents has high potential with regards to dynamic plant-adapted processes in the production of fruit and vegetables. To date, some applications have been tested that monitor the physiological state of horticultural products along the entire supply chain. This begins with plant-related production control, followed by an estimation of the optimum harvest time, up to fruit-dependent adjustments of optimal storage conditions.

According to the known issues of non-destructive spectroscopy, a new approach was figured out in the present work for the analysis of strongly coinciding spectral remission signals. The tool developed contributes to a more precise analysis of individual pigment contents, which vary during the cultivation of horticultural crops. Furthermore, the potential optical sensor can be applied without the need for re-calibration for different cultivars and seasonal effects. The tool is based on an iterative algorithm, which separates coinciding pigment spectra from *in-situ* as well as *in-vitro* readings from the sum spectrum of individual pigments. Finally, the algorithm was integrated into a stand-alone application containing a library of chlorophyll a (CHLa) and b (CHLb), as well as signatures of lycopene (LYC), β-carotene (bCAR), α-carotene (aCAR), lutein (LUT) and violaxanthin (VIO).

The new approach was initially validated through standardised spectrophotometric analysis of adjusted pigment compositions in comparison with established equation systems for calculating bCAR, LYC and total carotenoids (CAR_{total}). It was shown that the developed iterative multiple linear regression (iMLR) provides quantitative determinations of chlorophylls (r^2=1.00; $rmse$<8.88%) and high correlation also for the single carotenes LYC (r^2=0.99; $rmse$=5.03%) and bCAR (r^2=0.96; $rmse$=7.38%). In contrast to the other methods, iMLR was capable of determining the xanthophyll LUT (r^2=0.98; $rmse$=20.91%) in highly-spectral

overlapped mixtures of all pigments.

The iMLR was also applied on horticultural crops. A preharvest development of LYC, bCAR and LUT was monitored in tomato (*Solanum lycopersicum* L. cv. 'Counter') fruit. Considering the ripeness stages determined by the colour chart of the organisation for economic co-operation and development (OECD), the content of carotenes significantly increased during the fruit development on the plant. The content of bCAR and LYC ranged from 1.5 μgg^{-1} fresh weight (fw) in fruit of ripeness stage 4 up to 48.5 μgg^{-1} fw (stage 12) and 0.6 μgg^{-1} fw up to 132.4 μgg^{-1} fw respectively. By using the iterative MLR, LUT was measured in extracts of immature green tomato fruit. Its content gradually decreased from 19.7 μgg^{-1} fw to 11.5 μgg^{-1} fw. The following experiments were conducted on tropical fruit, which were particularly characterised by changing levels of carotenes and xanthophylls. In fruits of mango (*Mangifera indica* L. cv. 'Kent') a ripeness-related increase of bCAR and VIO content was observed. The mean VIO content of mango exocarp significantly rose from 8.63 to 9.41 μgg^{-1} dry weight (dw) in unripe and overripe fruit respectively. In contrast, the content of bCAR did not change significantly between unripe and medium ripe fruit, but was significantly increased in fully ripe fruit (16.50 μgg^{-1} dw). In the mesocarp of mango fruit VIO showed the highest increase during the study. In full ripe fruit the mean content was 24.71 μgg^{-1} dw. In contrast, the mean content of bCAR changed only slightly but significantly between stages of unripe and stored fruit. In regard to ripening papaya (*Carica papaya* L. cv. 'Hortus gold' and 'Solo') the use of time-series analyses and the separation of carotenes and xanthophylls also provided a differentiated insight into the fruit development. Since the mean content of bCAR was the highest in the exocarp of both cultivars, LYC was the quantitatively dominant pigment in the mesocarp of fruits. Significant changes of bCAR as well as LYC were measured after 74 and 26 hours of storage for the cultivar 'Hortus gold' and 'Solo' respectively. In contrast, no significant changes were found for VIO during the same period (unpublished results). Furthermore, the analysis of sweet cherries (*Prunus avium* L. cv. 'Schneiders späte Knorpel') rich in anthocyanins showed a significant decrease of LUT from 6 to 2.5 μgg^{-1} fw, but no significant change of bCAR (unpublished results).

In addition to spectral overlapping effects, caused by coinciding pigment absorption, strong influences of light scattering occur during the non-destructive readings. Due to this, a differentiated analysis of carotenoids became difficult. Attempts to correct the scattering in biological tissues of stone fruits have been studied using time-resolved measurements of photon-effective pathlengths in fresh and aging products. At least the spectral signal of single carotenoids could be separated from coinciding sum signals of pigment extracts by iMLR. Further non-destructively recorded spectra have been corrected through compensating for the disturbance caused by varying scatter coefficients.

In summary, it can be pointed out that individual pigment compositions are suitable indicators of the physiological stage of horticultural products. However, the spectral analysis of single pigment levels is challenging due to complex interactions of coinciding absorption and diffuse light scattering in natural pigment mixtures or in fruit extracts. Such varying spectral effects can already be observed in multi-component mixtures of plant extracts. From this, an improved method for the reliable decomposition of spectral signals was developed. It de-

termines single pigment contents in laboratory analysis of fruit and vegetables. Furthermore, extended technical approaches to estimate variable scatter effects in horticultural products could help to reduce spectral perturbations. Therefore more experiments have to be done on other organic materials. Finally, optical sensors, which have a high spectral sensitivity as well as suitable methods of signal analysis, need to be used to model robust calibrations of non-destructive readings of spectral remission in physiological plant properties.

Keywords:
iMLR, spectral analysis, pigments, tomato

Deutsche Zusammenfassung

Die Analyse veränderlicher optischer Pflanzenparameter unterliegt hohen technischen und methodischen Anforderungen. Durch die spektral überlagerte Lichtabsorption in natürlichen Multikomponenten-Gemischen und auf Grund der biologischen Variabilität des Pflanzenmaterials treten komplexe Störeinflüsse bei nicht-destruktiven Messungen an lebenden Produkten aber auch bei Referenzmessungen am Pflanzenextrakt auf. Dies ist während der Beprobung unterschiedlicher Sorten, in Abhängigkeit zur Anbausaison und sogar während der Entwicklung des Ernteprodukts bis zur Reife zu beobachten. Durch die Verfügbarkeit mobiler Sensoren und die Erforschung spektral-optischer Pflanzenparameter sind inzwischen reproduzierbare Messungen möglich. Vor dem Hintergrund der technischen und methodischen Fortschritte kann die optische Sensorik als konzeptioneller Teil für den Präzisionsgartenbau zur ökonomisch und ökologisch sinnvollen Nutzung natürlicher Ressourcen beitragen. Voraussetzung für eine robuste Kalibrierung zwischen zerstörungsfrei aufgezeichneten Spektren und physiologischen Pflanzenparametern sind jedoch optische Sensoren mit hoher spektraler Sensitivität und geeignete Analysemethoden der Messsignale.

Für die optische Sensorik im Präzisionsgartenbau spielen insbesondere farbgebende Pflanzeninhaltsstoffe (Pigmente) eine entscheidende Rolle. Sie sind auf Grund ihrer spektralen Absorptionseigenschaften durch Reflexions- oder Transmissionsmessungen im UV/VIS Bereich des elektromagnetischen Spektrums spezifisch adressierbar. Ausschlaggebend ist hierbei die Messung variierender Pigmentgehalte. Sie können als Indikator für den physiologischen Zustand von Blättern und Früchten dienen. Folglich hat die sensorische Pigmentbestimmung großes Potential für dynamisch an den Pflanzenzustand angepasste Produktionsmaßnahmen bei Obst und Gemüse. Inzwischen werden Anwendungen erprobt, die den physiologischen Zustand von gartenbaulichen Produkten in der gesamten Prozesskette überwachen können. Dies umfasst eine pflanzengerechte Anbausteuerung, die Bestimmung des optimalen Erntezeitpunktes und die Einstellung optimaler Lagerbedingungen.

In der hier vorgelegten Studie wurde ein neuer Ansatz zur Analyse spektral stark überlagerter und gestreuter Transmissions- und Remissionssignale erarbeitet. Hierfür wurde ein iterativer Algorithmus entwickelt, der *in-situ* und *in-vitro* aufgezeichnete Messsummensignale mit Hilfe von bekannten spektralen Pigmentsignaturen in Einzelanteile beteiligter individueller Pigmentabsorptionen zerlegen kann. Die Evaluierung erfolgte an verschiedenen gartenbaulichen Kulturen und an Pigmentextrakten. Der Algorithmus wurde schließlich in eine eigenständige Applikation integriert, die eine Datenbank der für die Validierungsversuche aufgezeichneten spektralen Signaturen der Chlorophylle a (CHLa) und b (CHLb), sowie Signaturen der Carotinoide Lycopin (LYC), β-Carotin (bCAR), α-Carotin (aCAR), Lutein (LUT) und Violaxanthin (VIO) enthält. Hiermit soll ein maßgeblicher Beitrag zur präziseren Laboranalyse veränderlicher Pigmentgehalte gartenbaulicher Kulturen unter praxisorientierten Bedingungen und ohne Neukalibrierung bei veränderlichen Fruchteigenschaften und saisonalen Effekten geleistet werden.

In weiteren Versuchen wurde die iterative multiple lineare Regression (iMLR) mit gebräuchlichen multivariaten Analysen verglichen. Die Experimente hierzu wurden an tropischen Früchten durchgeführt, die besonders durch veränderliche Gehalte an Xanthophyllen charakterisiert sind. Bei der zerstörungsfreien Analyse traten jedoch neben den absorptionsbedingten Überlagerungseffekten starke Einflüsse durch Lichtstreuung auf, die eine separierte Analyse von Carotinoiden erschwerte. Darauf aufbauend wurden Versuche zur Korrektur von reifebedingt variierenden Streukoeffizienten in Steinfrüchten veröffentlicht. Zunächst konnte hier der Gehalt individueller Carotinoide aus dem scheinbaren Messsummensignal der Pigmentextrakte durch den iterativen Ansatz separiert werden. Darüber hinaus wurden zerstörungsfrei aufgezeichnete Spektren mit Hilfe von zeitaufgelösten Messungen der tatsächlichen mittleren Photonentransportwege gegen variierende Streueinflüsse korrigiert.

Zusammenfassend ist anzumerken, dass besonders die Veränderung individueller Pigmentgehalte ein geeigneter Indikator zur Beschreibung des physiologischen Zustandes gartenbaulicher Produkte ist. Die spektrale Analyse von Einzelpigmentgehalten wird jedoch in nativen Gemischen oder auch im Extrakt durch ein komplexes Zusammenwirken von überlagerter Absorption und diffuser Lichtstreuung erschwert. Variierend überlagernde Absorptionen existieren auch im Pflanzenextrakt bei Mehrkomponentengemischen. Daher wird in zahlreichen obstbaulichen Laboren ein verbessertes Verfahren für eine zuverlässige Zerlegung der spektralen Informationen in pflanzliche Einzelpigmentgehalte benötigt. Hier kann der beschriebene methodische Ansatz einen Beitrag für präzisere Analysen leisten. Darüber hinaus können erweiterte technische Ansätze zur Erfassung veränderlicher optischer Streueigenschaften gartenbaulicher Produkte mit Hilfe zeitaufgelöster Messungen helfen, spektrale Störeinflüsse zu reduzieren. Hierfür sind Versuche an weiteren Kulturen durchzuführen.

Schlagwörter:
iMLR, Spektralanalyse, Pigmente, Tomate

Contents

List of publications included in this thesis

This thesis is based on the following articles, which are referred to in the text by their Roman numerals (I – III). The articles were modified according to the thesis layout.

(I) Pflanz, M. and Zude, M. (2008). Spectrophotometric analyses of chlorophyll and single carotenoids during fruit development of tomato (*Solanum lycopersicum* L.) by means of iterative multiple linear regression analysis. *Applied Optics*, 47(32):5961-5970.

(II) Pflanz, M.; Mudau, N. and Zude M. (2010). Separation of absorption coefficients from ripeness-related fruit pigments in stored mango. *Erwerbs-Obstbau*, 52(1):1-9.

(III) Zude, M.; Pflanz, M.; Spinelli, L.; Dosche, C.; Torricelli, B. (2011). Non-destructive analysis of anthocyanins in cherries by means of Lambert-Beer and multivariate regression based on spectroscopy and scatter correction using time-resolved analysis. *Journal of Food Engineering*, 103(1):68-75.

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Abbreviations

Pigments

ANT_{total}	total Anthocyanins
aCAR	α-Carotene
bCAR[1]	β-Carotene
CAR_{total}	total Carotenoids
CHLa	Chlorophyll a
CHLb	Chlorophyll b
bCRY	β-Cryptoxanthin
CYA	Cyanidin
LUT	Lutein
LYC	Lycopene
NEO	Neoxanthin
PEL	Pelargonidin
PEO	Peonidin
VIO	Violaxanthin
XAN_{total}	total Xanthophylls
ZEA	Zeaxanthin

Spectral indices

I_{NChl}	normalised chlorophyll index
I_{NA}	normalised anthocyanin index
IP	inflection point
NAI	normalised anthocyanin index
NDVI	normalised difference vegetation index
RVSI	red-edge vegetation stress index

[1]Differing to the abbreviation CAR for β-Carotene in Paper I and II

others

c	concentration
CIELAB	CIE 1976 ($L*$, $a*$, $b*$) colour space
cv.	cultivar
DTOF	distribution of time-of-flight
dw	dry weight
equ.	equivalent
fw	fresh weight
HPLC	high performance liquid chromatography
iMLR	iterative multiple linear regression
IRF	instrument response function
k	specific extinction coefficient
LC	liquid chromatography
LV	latent variable
MLR	multiple linear regression
MSC	multiplicative signal correction
	equivalent to: multiplicative scatter correction
N	refractive index
NIR	near-infrared
OECD	organisation for economic co-operation and development
PCA	principal component analysis
PLS	partial least squares
(%)R	(relative) spectral reflection
RGB	colour model of red, green and blue
$\%\,rmse$	(relative) root mean square error
rmsec	root mean square error of calibration
SEP	standard error of prediction
SNV	standard normal variates
SP	spectrophotometry
SSC	soluble solids content
TA	titratable acidity
TIRF	total internal reflectance fluorescence
TLC	thin layer chromatography
Tr	spectral transmission
UV	ultraviolet
VIS	visible

1. Introduction to precision horticulture

1.1. Advanced phytomonitoring technologies

Horticultural crops are edible parts of living plant – apart from ornamentals – which are exposed to series of intensive pre- and postharvest treatments. Along the supply chain up to the point of sale, the quality of horticultural products is mainly affected by cultivation conditions, maturity at harvest, and postharvest handling. But in spite of technological advance during the last decades many decisions within the different stages of production are still subjectively made, predominantly through the experience of growers and retailers.

However new technologies are recently developed and omnipresent in discussion as precision farming or precision horticulture concepts. Regarding this the horticultural production should be adapted and controlled by an intelligent crop monitoring that improves the process management (Bakker, 1995; Schmidt et al., 2008; Ruiz-Altisent et al., 2010). Instruments for on- and off-plant fruit monitoring offer precise and objective methods for the prediction of optimum harvest date as well as fruit grading during postharvest chain (Brezmes et al., 2000; Zude and Herold, 2002; Walsh et al., 2004; Herold et al., 2005). The targets are an economised input of resources, as possible adapted to the requirements of crops (Langhans et al., 1981; Hurd and Graves, 1984) and a minimisation of losses in quality due to decay of perishable commodities after harvest (Kader, 2002; Zude, 2009). Such rethinking is consistent with the current situation of globalisation where the competition is rising, and increasing prises for energy lead to higher operational costs. Additional, changes in consumer preferences induce an increasing demand in sustainably-produced commodities and high-grade, healthy food.

Even though the demand for premium food is rising, most consumers are not willing to pay more money for higher quality. Consequently, retail is exerting pressure on producers to provide food with a long shelf life and low prices (Kress and Brimelow, 2001). Nevertheless, fruit and vegetables minimum quality attributes should be guaranteed. That primarily involves the appearance (including shape, colour, and defects), size, flavour, taste and texture (Chen and Sun, 1991; Abbott, 1999). Further, changes in chemical constituents, especially metabolites of the secondary pathway, and losses in vitamin content influence the nutritional value of horticultural products (Kader, 2002; Huyskens-Keil and Schreiner, 2003). Even if quality levels are appraised differently according to preferences of producers, retailers and consumers (Shewfelt, 1999), in the end, the consumer's acceptance is certainly the determining factor in the composition of a diet (Opara et al., 2007).

However, the human understanding of quality is quite abstract. Everyone has her or his own individual criteria that are often prejudiced by individual preferences or expectations (Abbott, 1999). Moreover certain attributes like smell, taste or even colour are difficult to communicate. Here, instrumental techniques offer physical parameters, objectively to quantify and comparable for researchers, industry and consumers (Norris, 1983; Abbott, 1999). It has been further shown for decades that a wide range of innovative sensory technologies, many of them non-destructive techniques, may be feasible for horticultural applications (Chen and Sun, 1991; Zwiggelaar, 1998; Butz et al., 2005; Ruiz-Altisent et al., 2010).

In this regard, optical instruments provide useful information about valuable plant and fruit properties, which can be non-destructively recorded, reproduced, and precisely analysed even with relation to hidden attributes. For this purpose, spectroscopic techniques are suitable instrumental methods to address varying contents of biochemical compounds in horticultural commodities by means of VIS and near-infrared (NIR) light transmission, reflectance, or fluorescence (Birth et al., 1957; Belton, 1997; Lichtenthaler and Buschmann, 2001; Merzlyak et al., 2003b; Solovchenko et al., 2010). Innovative, inexpensive and moreover portable spectrophotometer devices have been developed and are now available for effective on-field monitoring of multitudinous plant and fruit attributes as well as in postharvest chain (Truppel et al., 2000; Di Natale et al., 2002). Additionally, a rapid increase of powerful computing affords complex plant models and advanced signal processing methods (Naes et al., 1990; Ni and Gong, 1997; Mizrach et al., 1999; Westerhuis et al., 2001; Martens et al., 2003; Zude, 2003; Janik et al., 2007).

Further, non-destructive texture analyses have been developed to estimate fruit flesh firmness by VIS and NIR spectroscopy (Isaksson and Griffiths, 2002; Subedi and Walsh, 2009). In terms of on-field fruit monitoring, maturity-dependent soluble solids contents (SSC) of apple and citrus fruit have been predicted non-destructively by portable spectroscopy techniques using spectral readings of partial light transmittance (Miller and Brown, 2004; Zude et al., 2006). Under postharvest conditions, non-destructive NIR spectroscopy has been applied to estimate varying contents of sugars, dry matter and SSC in apples, citrus, and mango fruit as well (Walsh et al., 2004; Guthrie et al., 2005; Delwiche et al., 2008). Also, scattering image processing techniques have been figured out to estimate the SSC of apple fruit non-destructively after harvest (Lu, 2004; Lu, 2007). Here, a novel laser-induced backscattering technique was used, subsequently confirmed by a close relationship between NIR light scattering measurements of apple and kiwi fruit tissue and its maturity (Qing et al., 2008b; Baranyai and Zude, 2009).

In terms of commodity-specific hue, plant pigments directly affect the appearance of vegetables and fruit and can even be observed by human senses. Pigments consequently influence the product acceptance of consumers. Furthermore, changes in pigments play a key role in fruit development due to their relation to physiological changes of fresh products (Knee, 1972). Detectable early by optical instruments, varying contents of chlorophylls, anthocyanidins, and carotenoids should be used to determine the quality of horticultural products (Norris, 1983). The loss of green colour induced by a decrease of CHLa and CHLb is well

correlated with senescence of plant tissue (Matile et al., 1999) and fruit aging respectively (Watada et al., 1984; Brady, 1987; Kader, 1999; Prasanna et al., 2007). Carotenes such as aCAR and bCAR, LYC, or numerous xanthophyll esters vary in relation to fruit genome in general. Additionally, individual changes of carotenes have been detected in climacteric fruit according to maturity as well as under varying environmental conditions in preharvest and storage conditions in postharvest chain (Cano and Deancos, 1994; Mercadante and Rodriguez-Amaya, 1998; Kozukue and Friedman, 2003; Kuti and Konuru, 2005; Ornelas-Paz et al., 2007). Changes in anthocyanins in non-climacteric fruit have been also found to be related to ripeness at harvest and storage conditions (Sass-Kiss et al., 2005; Wrolstad et al., 2005; Goncalves et al., 2007).

While chlorophylls are primary involved in growth, development, or reproduction of an organism, carotenes, xanthophylls and anthocyanins are secondary metabolites, which are important for plant defence and their antioxidative potential also makes them important for the human diet (Stahl and Sies, 2002). In terms of their provitamin A activity it was shown that a diet rich in carotenoids prevents chronic diseases such as age-related macular degeneration (Landrum and Bone, 2001) and certain cancers (Franceschi et al., 1994; Levy et al., 1995; Giovannucci, 2002). With regard to a high level of consumption (e.g. of tomatoes), well-graded food is important for a balanced diet all over the world. So it is reasonable that the health promoting properties of carotenoids have intensified scientific and commercial research concerning carotenoid biosynthesis in fruit and vegetables since the last three decades. The grading of food could further lead to better consumer acceptance, ensuring humanity's nutrition and also reaching a higher market value. According to the quality of food, pigments are bioactive compounds as well as predictive biomarkers, which can be sensitively detected through non-destructive spectroscopy. New innovative optical instruments purpose a continuous monitoring of main pigments such as chlorophylls, carotenoids and anthocyanidins. Their implementation should offer valuable information about physiological product properties, essential for improving cultivation processes and avoiding losses along the postharvest chain.

Nevertheless, due to a complex spectral interaction of numerous individual pigments in living horticultural products, advanced *in-situ* analyses are needed to separate coincided signals corresponding to changes in certain chlorophylls, carotenes and xanthophylls (see chapter 2).

1.2. Perspectives on plant-related environmental control

Variations in the quality of horticultural products may basically be determined by the plant genome, but are also affected by environmental factors during the whole cultivation chain. In preharvest, plant and fruit development is subjected to changes in water and dry matter accumulation as well as varying biochemical and mineral compounds. That means a continuous recording of indicative data, which correspond to plant responses to changing environmental

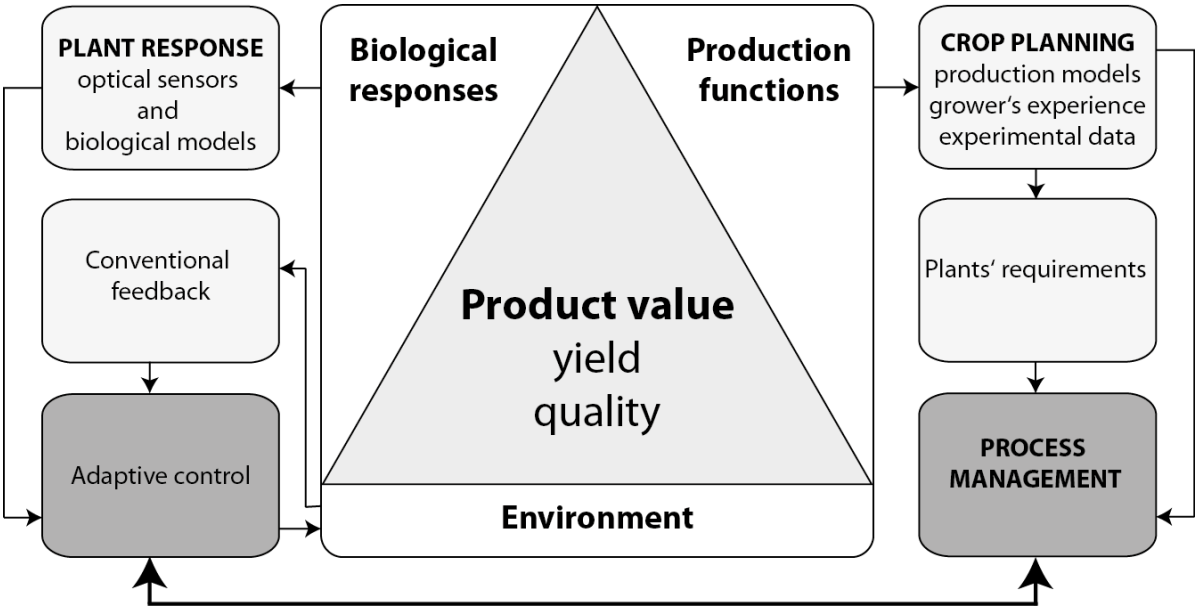


Figure 1.1.: In terms of phytomonitoring techniques the integration of optical sensors for measuring plant responses provides an improved process management through the hole product chain (modified from Sigrimis et al., 1999). Product quality and yield should be the main target values for optimisation.

conditions, might be used to improve cultivation management and consequently the quality of products (Figure 1.1).

In ordinary greenhouse production, approaches to environmental optimisation were to date mainly limited to the measurement and control of air temperature, relative humidity and CO_2 concentrations (Chalabi et al., 1996; Sigrimis and Rerras, 1996; Ioslovich and Seginer, 1998). More innovatively, leaf temperature, sap flow rate, and fruit variation have been monitored (Jackson et al., 1981; Steinberg et al., 1988; Huguet et al., 1992). Furthermore, approaches to plant-related direct feedback control had been developed a long time ago (Hashimoto et al., 1981; Hashimoto et al., 1985; Challa and Van Straten, 1993; Tantau, 1993). Here, environmental factors are considered to be the input and plant properties the resulting responses. Such approaches are known as phytomonitoring approaches according to the vision of "speaking plant" (Udink ten Cate et al., 1978).

Since robust and low cost sensor systems are of widespread availability, valuable information about plants' physiological stage can be measured more precisely. Feasible feedback chains have been identified through measuring physical parameters that are well correlated with photosynthesis efficacy, stomatal transpiration dynamics, and leaf-air temperature difference (Field et al., 1989; Millan-Almaraz et al., 2010). Significantly affected by environmental conditions, these properties afford real-time decisions about optimal cultivation management (Sigrimis et al., 1999; Morimoto and Hashimoto, 2000).

In order to accomplish that, plant-selective gas exchange measurements on leaves provide

a direct determination of photosynthetic efficiency. Here, fast plant responses have been detected according to varying air temperature, relative humidity and CO_2 concentration in greenhouses (Schmidt et al., 2008). Learning from this, an early detection of microclimatic changes could help prevent fungal diseases, further improve physiological status by higher stomatal conductance and save energy by a precise adjustment of heating, cooling and electric illumination (Schmidt, 1998; Prenger et al., 2002; Ruhm et al., 2009). In this context, Ruhm et al. (2009) proposed to measure the temperature of plant tissue to calculate the thermal balance of a whole plant population. Intelligent approaches based on plant responses were also proposed to delay the ripening of tomatoes through an optimised heat treatment (Morimoto and Hashimoto, 2000). More advanced applications implement offline crop water stress detection based on transpiration efficiency analysis, which led to intelligent scheduling of irrigation according to speaking plant concepts (Escalona et al., 2000; Ton et al., 2004).

In order to avoid qualitative product losses, the composition of individual leaf pigments should be balanced to achieve optimal photosynthetic requirements in terms of various growth conditions to which plants are exposed.

1.3. Measuring fruit responses to pre- and postharvest processes

1.3.1. Changes in individual pigments during fruit maturity and ripeness

During the genetically programmed fruit development, a series of some major organoleptic, physiological and biochemical changes such as increased respiration rate and activity of cell wall degrading enzymes, and a transient increase in ethylene production have been determined (Brady, 1987; Prasanna et al., 2007). Fully mature fruit are generally characterised by reduced fruit flesh firmness, and edible ripe fruit with desirable quality attributes related to SSC, titratable acidity (TA) and firmness (Shewfelt, 1999; Opara, 2000; Kader, 2002; Zude, 2009). In many cases, an increased antioxidative potential can also be observed according to higher mean retention time for fruit development on the plant (Dorais et al., 2008). Further, significantly increased contents of vitamins have been found in numerous horticultural products, related to the fruit maturity at harvest (Betancourt et al., 1977; Mercadante and Rodriguez-Amaya, 1998; Kader, 1999; Frenich et al., 2005).

The evidence is that the harvest date needs to be set precisely between the times when fruit are still immature, and become overripe and consequently susceptible to decay. Here, the variability of individual pigments would be well suited for *in-situ* plant monitoring or indication of the fruit maturity stage (Table A.1 and A.2). However, an obvious simply colour change during the fruit development occurs due to a complex interaction of numerous

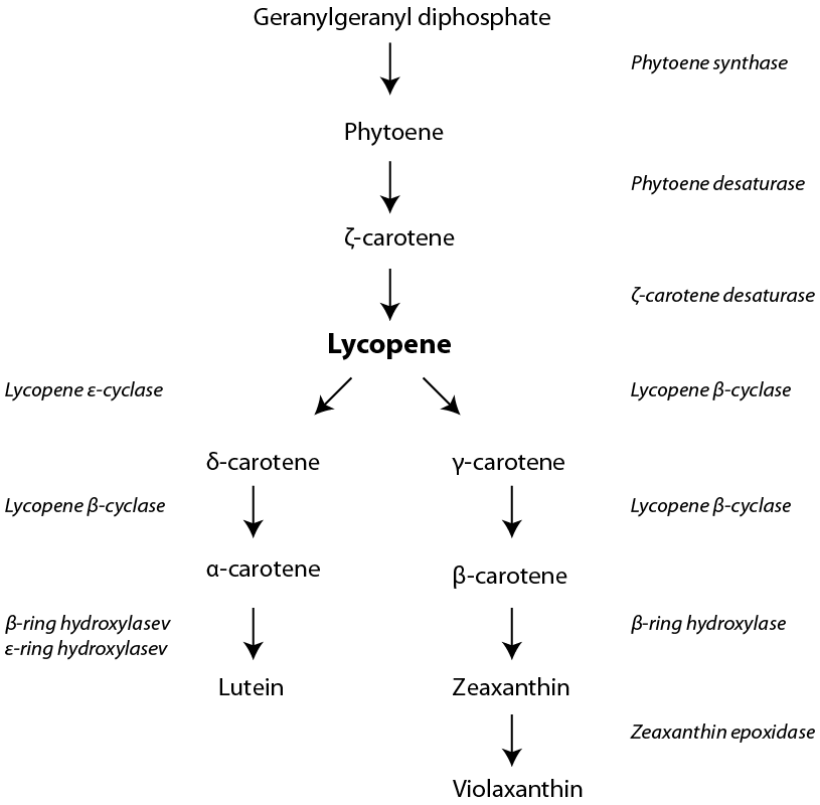


Figure 1.2.: Carotenoids biosynthesis pathway in plants and fruit (generalised and modified from Britton, 1993; Hirschberg, 2001 and Sandmann, 1994)

pigment groups (Table 1.1). In general, individual variation of pigments as well as their ratios to one another are important physiological attributes that closely correspond to the ripeness stage, various damages and disorders (Gross et al., 1978; Knee et al., 1989; Abbott, 1999; Herold et al., 2005). In a more detail, during the fruit development (Figure 1.2), a loss of green colour through chlorophyll decomposition is accompanied by an increase in bright yellow and red colours due to biosynthesis of carotenoids (Stiles, 1982; Webb, 1985). In xanthophyll-containing fruits, ripening is further accompanied by a decrease in carotenes simultaneous with a rapid increase in the content of xanthophylls (Rodriguez et al., 1976; Gross, 1987). Basically the characteristic green of immature fruit is based on variable amounts of CHLa and CHLb (Blanke and Notton, 1992). Although the alternation of green and yellow colours in senescent plant tissue is a known phenomenon, the whole cascade of chlorophyll degradation is largely unexplained. Indeed, it has been shown by Matile et al. (1999), that within senescence, a dismantling of pigment-protein complexes in the thylakoid membranes affords an achromatic breakdown product of chlorophyll which has optical unmasking effects on other chromophoric compounds. In addition to this, an accumulation of anthocyanins in vacuoles and a retention of carotenes such as aCAR, bCAR, LYC and xanthophylls such as LUT and VIO let the fruit colour change from green to yellow or red (Tucker and Grierson, 1987; Lizada, 1993). In tomatoes an inhibited biosynthesis of some carotenes (aCAR, bCAR, γ-, ζ-carotene) was detected by Koskitalo and Ormord (1972) after the colour of ripening fruits changed from orange to red.

The changes in colour of ripening tomato fruit due to varying single carotenoids have also been shown in Paper I (Pflanz and Zude, 2008). The investigated change of pigment contents in tomato fruit resulted in no significant increase of bCAR content in fruit, which would have exceeded the breaker-turning stage. In contrast, the content of LYC increased continuously up to full ripeness. A significant content of LUT was also detected in green fruit before turning from yellow to red.

Knee (1972) observed that certain carotenes and xanthophylls vary highly in apple fruit depending on maturity. Moreover, differences in carotenoid fractions have been determined to be related to fruit cultivar. In apple fruit that includes various ratios of VIO, neoxanthin (NEO), bCAR, and LUT (Galler and Mackinney, 1965). Variable compositions of bCAR, NEO and VIO have also been found in ripening mango fruit observed by chromatographic methods (Sadana and Ahmad, 1949). Here, an increase in bCAR and less itemised xanthophylls was reported. Jungalwala et al. (1963) found 16 different carotenoids in fully ripe 'Alfonso' mango fruit and figured out that their distribution during ripening plays an essential role in the carotenogenesis. A general hydrolytic degradation of bCAR was discussed (Goodwin, 1971; Sandmann, 1994; Hirschberg, 2001; Bramley, 2002) and detected in pepper and tomato fruit as well, leading to enhanced contents of β-cryptoxanthene (bCRY), zeaxanthene (ZEA) and VIO (Fraser et al., 1994; Delgado-Vargas et al., 2000).

Table 1.1.: Preliminary list of studies and analysis methods with reference to varying pigments in different ripening fruit. Ripeness-related changes of apple fruit pigments were not investigated in this thesis, but also shown due to their scientific and economic relevance.

Crop	Cultivar	Pigment (trend)	Non-destructive analysis	Reference method	Remarks	References
Tomato	Ailsa Craig	bCAR (++) LUT (-) LYC (++) NEO (-) VIO (o)	colour appearence	HPLC	individual change from mature green to ripe fruit	(Fraser et al., 1994)
	Momotaro	bCAR (+) LYC (++)	–	HPLC	different development from 50th day after flowering	(Kozukue and Friedman, 2003)
	Capita F1	bCAR (+) LUT (o) LYC (++)	colour chart	HPLC	different spatial distribution in ripening fruits	(Polder et al., 2004)
	Lemance F1	LYC (++)	CIELAB	TLC	correlated with carbohydrate content	(Helyes et al., 2006)
	Cervil	bCAR (+) LYC (++)	CIELAB	HPLC	bCAR synthesis triggert by increasing LYC	(Gautier et al., 2008)
	Jennita	bCAR (o) LYC (++)	colour chart	HPLC	significant differences in terms of harvest season	(Slimestad and Verheul, 2005)
	Laura	LYC (++)	CIELAB	HPLC	correlation to a*/b* ratio	(Arias et al., 2000)
	Cerasiforme	bCAR (+) LUT (+) LYC (++) NEO (o) VIO (+)	colour chart	TLC	different distribution in pulp and flesh	(Laval-Martin et al., 1975)
	n.s.	bCAR (+) LYC (++) XAN_{total} (o)	–	TLC	individual development	(Kuhn and Grundmann, 1932)
	Walter	bCAR (o) LYC (++) XAN_{total} (o)	–	HPLC	no significant changes of bCAR at early ripeness stages	(Watada et al., 1976)
	Early Red Chief	bCAR (o) LYC (++)	Hunter LAB	TLC	bCAR synthesis stopped after colour changed from orange to red	(Koskitalo and Ormrod, 1972)
	Moneymaker	bCAR (+) LYC (++)	Hunter LAB	HPLC	exponential increase	(Giovanelli et al., 1999)

Continued on next page

Table 1.1 – continued from previous page

Crop	Cultivar	Pigment (trend)	Non-destructive analysis	Reference method	Remarks	References
Mango	Keitt	bCRY (o) bCAR (++) NEO (o) VIO (++)	colour appearence	HPLC	individual carotenoid compositon	(Mercadante and Rodriguez-Amaya, 1998)
	Cogshall	CAR_{total} (++)	–	LC	no individual change of carotenes or xanthophylls	(Joas et al., 2012)
	Badami	bCRY (+) LUT (-) ZEA (+)	–	HPLC	time depended individual changes	(John et al., 1970)
	Dashehari	bCAR (+)	–	LC	significant changes in terms of harvest date	(Kalra and Tandon, 1983)
	Chok Anan	bCAR (+) trans-bCAR (o)	CIELAB	HPLC	significant differences of individual carotenes	(Kienzle et al., 2011)
	Nam Dokmai	bCAR (++)	CIELAB	HPLC	corralated to CIELAB colour	(Mahayothee et al., 2007)
	Chounsa Desi	bCAR (++) NEO (+)	–	TLC	ripeness-related shift of carotenoid profiles	(Sadana and Ahmad, 1949)
Cherry	Schneiders Späte Knorpel	aCAR (+) CAR_{total} (o) CYA equ. (++)	UV/VIS SP	LC	ripeness-depending changes	(Zude et al., 2011)
	Burlat Summit	ANT_{total} (++) CYA (++) PEL (+) PEO (o)	CIELAB	HPLC	significant differences in development of individual anthocyanins	(Goncalves et al., 2004)
Apple	Cox's Orange	bCAR (o) LUT (o) NEO (o) VIO (o) XAN esters (++)	–	LC	significant different changes of individual carotenes and xanthophylls	(Knee, 1972)
	Jonathan	ANT_{total} (+)	–	LC	accumulation in fruits not being immature yet	(Chalmers et al., 1973)

not specified (n.s.); degradation (-); no significant changes (o); slight increase (+); high accumulation (++)

Attempts to estimate the accumulation rate of red pigments (anthocyanins) in apple skin for maturity prediction have also been reported for a long time (Chalmers et al., 1973). But various levels of anthocyanin could not be separated from seasonal variations. Furthermore, a relationship between degradation and synthesis of anthocyanins was observed in immature fruit at varying light and environmental conditions (Creasy, 1968; Chalmers et al., 1973).

In contrast, the amount of total anthocyanins (ANT_{total}) in cherries is usually higher in ripe fruits than in partially ripe ones (Goncalves et al., 2007). More specifically, in freshly harvested, fully ripe cherries, the levels of cyanidin-3-rutinoside were found to represent 63–94% by weight of the ANT_{total} (Mozetic et al., 2006). Considering this, changes in anthocyanin accumulation might be used as an indicator of the maturity of horticultural products with regard to the variety and variations in the season.

Due to the fact that the content of pigments, and consequently their composition within a fruit, varies in many cases, single pigments are suitable parameters for an objective detection by optical instruments. In contrast, conventional methods of colour measurement using colour cards are erratic and less sensitive to slight colour variations. Non-destructive spectral techniques provide a significant contribution to the quality assessment of horticultural products. The results are objective, the measurements can be performed at low costs and in large numbers, they are repeatable, reproducible, and its objective character leads to a high level of acceptance in academia, industry and ultimately the consumer market. A more precise quality determination can only be achieved through costly and time-consuming chemical analyses.

In conclusion, non-destructive techniques are a perfect fit for an accompanied monitoring of cultivation with respect to quality control, cultural conditions, optimum harvest time, postharvest storage and quality-related sorting. And at an advanced stage, these technologies can help make production processes more efficient without a loss of product quality by keeping costs moderate and saving resources.

1.3.2. Fruit pigment compositions in different environmental conditions

Agronomic practices have a significant influence on plant growth and thus on the nutritional value of fruit. But in terms of their environment-dependent development predominantly major chromophors like anthocyanidins and carotenoids have been investigated (Table A.1 and A.2). In contrast to, it is shown that individual carotenes like bCAR and LYC in tomatoes as well as xanthophylls like VIO and bCRY in mango fruit were found to be significantly variable according to the environmental conditions in pre- and postharvest (Mercadante and Rodriguez-Amaya, 1998; Kozukue and Friedman, 2003; Kuti and Konuru, 2005). It was further concluded that different types of carotenoids in apple fruit may vary individually due to photooxidative radiation intensity and duration (Felicetti and Schrader, 2009). In addition,

numerous studies have dealt with varying adjustments to artificial radiation, water management, mineral nutrients, growing systems, and stage of fruit development on antioxidants and other compounds of value to human health (Davies and Hobson, 1981; Hobson, 1988; Thakur et al., 1996; Dorais et al., 2001; Dumas et al., 2003; Collins et al., 2006; Lester, 2006; Dorais, 2007). Consequently, an evaluation of those crop management strategies should be challenged, whose combinations maximise the physiological development of fruit, considering the individual accumulation of fruit pigments at preharvest stages. Furthermore the change of pigments in fruit (Table A.2) should be used as an output parameter for controlling postharvest storage conditions (De Baerdemaeker and Hashimoto, 1994). Here, intelligent fruit monitoring may assist in defining the conditions required through the use of optical sensors.

However, an implementation of phytomonitoring technologies to improve horticultural cultivation involves a certain amount of risk in terms of fruit quality. If environmental conditions are adjusted only in focusing on a "well being" of plants, the biosynthesis of valuable compounds in fruits might be reduced in certain cases. Under suboptimal conditions for high growth rate, plants accumulate indeed more protective pigments and antioxidants in tissue (Laval-Martin et al., 1975; Léchaudel and Joas, 2007). Triggered by biotic or abiotic stresses, the biosynthesis of carotenes, xanthophylls and anthocyanins, their composition and distribution in fruits is closely regulated during development and responsive to environmental stimuli (Britton, 1993; Pogson et al., 1996; Welsch et al., 2000; Hirschberg, 2001). Those include deficits in water (drought), unbalanced salinity, extremes and sudden changes of temperature, oxygen deficiency in soil due to flooding, and photo-oxidative stress through exposure to high light intensity in the photosynthetically active part of the UV-A, UV-B and VIS spectrum (Table A.1).

1.4. Parameter fusion

In advanced technologies for cultivation, controlling a large amount of plant and climate sensors provides a host of measurable parameters. Such various information only contains a subset of parameters suitable for processing by or presenting to human supervisors. Consequently, it appears reasonable to combine certain measured values through sensor integration especially in real-time monitoring systems.

In fruit monitoring, as part of a complex controlling system, sensor fusion approaches could improve the robustness of fruit properties modelling. It has been shown by Steinmetz et al. (1999) that the standard error of prediction (SEP) for non-destructively measured sugar content in 'Golden Delicious' apples was lower after combining colour and spectrophotometrically NIR readings (770-1070 nm), than the SEP for each parameter alone. Because of a non-linear correlation between colour and sugar content, a multilayer neuronal network was used to fuse averaged R (red) and B (blue) values from RGB colour readings with two latent variables (LV) from NIR readings after principle component analyses.

A fusion of firmness, colour and acidity values through the use of neuronal networks was applied to improve the quality assessment of tomato fruit by colour categories (Shmulevich et al., 1994). However, the error of classification amounted to 12%. Ozer et al. (1995) combined data of destructive (firmness) with non-destructive (colour) sensors and could improve the error classification rate when applied on melons. Different methods of acoustic resonance analysis were compared with classifying fresh peaches into soft, ripe and immature through non-destructively predicted firmness (Armstrong et al., 1997). For this purpose eight parameters were determined from acoustic spectra by fast fourier transformation and linear regression. It was further shown, that if the mass of samples is included into the linear regression analysis, the adjusted coefficient of determination (adj. r^2) will significantly increase. The fusion of data obtained with electronic nose and spectrophotometric readings improved the prediction of the fruit quality by means of outer product analysis (Di Natale et al., 2002).

Schmidt (1998) developed an integrative low-cost system, which combines a series of maintenance sensors. Air temperature, relative humidity, CO_2 concentration and the area of leaves was measured inside of an attached cuvette, referenced to greenhouse air conditions and finally combined to a model of plant transpiration rate. By using several leaf sensors, an integrated plant mapping of the whole crop surface was achieved and used for automatical greenhouse climate control.

In terms of varying optical fruit properties in individual pigment contents, multivariate models describing the time of flight of photons through biological tissue (Zude et al., 2011) may be combined with additional data obtained by absorption properties by means of MLR methods developed in the present study (Pflanz and Zude, 2008).

1.5. Hypotheses and objectives

The main objective of the experiments described in the present work was to validate a new methodical approach of analysing spectral-optical readings according to ripeness-related variations in native fruit pigments. By means of spectroscopy or multi-spectral methods, monitoring the development of single carotenes like LYC, bCAR and LUT in tomatoes or xanthophylls like bCRY and VIO in mangoes as well as papaya fruit could give more precise information about the maturity stage of fruit than colour readings or contents of CAR_{total}. Therefore, optical measuring techniques – established in research – were used to determine the change in spectral characteristics of partially transmitted or reflected UV and VIS radiation.

However, all the key benefits of spectral techniques are restricted by a complexity of interpretation due to coinciding and scatter-influenced signals. Spectral information includes the absorption of all native absorbers in the fruit. The spectral separation of each pigment absorption would enable the analyses of single pigments. Furthermore, the spectral signature of *in-situ* measured plant material varies not only due to the pigment contents, but also

due to biological variability like ripeness-dependent changes of texture and water content. The latter phenomenon leads to varying light scattering properties. Such perturbations appear when comparing samples from different cultivars and seasons, and even during the fruit development. A partly unknown interaction between chemical and optical properties of living plant material results in unstable calibration models with high discrepancies between predicted and measured quality parameters. For an approach to improve spectral-optical sensors providing more robust calibration models, the main interest of the present work was focussed on the phenomena of spectral interference of different light absorber. This occurs if more than one chromophor as constituent is present in a mixture of extracts from plant tissues or during non-invasive spectral analyses. As a consequence of such simultaneous multiple absorption events, the signal that can be measured by instruments is a sum signal of all compounds. Multivariate statistics, such as iMLR could help to separate signals, which are significantly correlated to varying single pigment contents from partially coincided destructive and non-destructive spectral recordings. At this a stepwise recomposing of UV-VIS recorded sum signals through the use if specific absorption signatures could help determine the single chromophors involved in the maturity processes of particular samples. In detail, the ripeness-related variation of CHLa and CHLb, bCAR, aCAR, LYC, LUT and VIO was subject to investigation in this work. This technology has not been published about so far.

In terms of measurement uncertainties caused by optical perturbations during the fruit development, the new approach of iterative spectral analysis has to be compared with established analytical methods of quantifying chlorophylls and carotenoids after chemical extraction. For this purpose, spectral profiles of typical carotenoids and chlorophylls should be made from high-grade pigment standards, which are also required for calibrating liquid chromatography (LC) analyses. With regards to its occurrence in biological tissues coinciding light absorption has to be reproduced from predefined multi-component mixtures and separated consequently into its spectral constituents by the new approach. Next to this, the iMLR has to be validated based on spectral readings on fresh fruit and its pigment extracts. For this purpose, the development of chromophores has to be monitored non-destructively at different ripeness stages on plant and at adjusted storage conditions after harvesting. However, since their contents of carotenoids and chlorophylls vary widely between different fruit ripeness stages, tomatoes are suitable samples and have to be investigated for discussing perspectives of advanced and long-term concepts for greenhouse control through non-destructive sensor technologies (Paper I).

In addition to the experiments to be done on artificial pigment mixtures and model fruit, the new approach has to be tested further on fruit with divergent spectral profiles. For this, tropical fruit like mango and papaya should be adequate samples showing differences in the development of chromophoric constituents to that of tomatoes during the fruit maturity. In particular the analysis of xanthophylls, which are subsequent metabolites of the lycopene biosynthesis pathway, should demonstrate the benefits of applying the iMLR to a wide range of horticulture products. Cooperations with research institutions in South Africa (Department of Plant Production, University of Limpopo and Department of Horticultural Science, University of Stellenbosch) will enable these experiments (Paper II).

To what extent the variation of light scattering interact with non-destructive spectral readings within the fruit tissue of ripening cherries, and how these influences might be reduced through distributed time of flight measurements (DTOF) will be shown in Paper III. In contrast to commonly used approaches of modeling the effective path length of photons based on theoretical assumptions, in the following experiments the diffuse light distribution has to be determined by time resolved analysis. If optical perturbations can be reduced regarding their spectral sum signals, the new approach of iMLR should separate single constituents from the divergent spectral profile also from non-destructive readings on cherry fruit.

2. UV/VIS spectral analysis

2.1. Coinciding light absorption and scattering in biological tissue

2.1.1. Non-destructive spectroscopy on intact fruit and vegetables

The needs for research of monitoring technologies on intact fruit and vegetables are mainly promoted by producers improving their economic success, but also through increasing consumer awareness for high-grade and healthy fresh products. For this reason, for decades several approaches have been focused on correlations between physical properties and physiological stages of horticultural products. Particularly, spectrophotometric techniques were shown to have great potential for non-destructive quality evaluation on specialty crops at pre- and postharvest (Chen and Sun, 1991). In terms of optical instruments, the interaction of chlorophylls, carotenoids and anthocyanidins with the electromagnetic radiation has been figured out to be detectable by spectral readings at UV/VIS wavelength ranges (Hilbert and Jansen, 1934; Miller, 1937; Mackinney, 1941; Comar and Zscheile, 1942; Zscheile and Porter, 1947; Herschberg and Sixma, 1962a; Herschberg and Sixma, 1962b; Butler, 1964; Birth, 1979; Porra et al., 1989). This provides an *in-situ* estimation of maturity and is consequently useful for the prediction of the optimal harvest date according to the pigment contents in the intact biological tissue (Knee, 1972; Massie and Norris, 1975; Watada et al., 1976; Meister, 1977; Birth, 1979; Nattuvetty and Chen, 1980; Lichtenthaler et al., 1996; Gitelson et al., 2002; Merzlyak et al., 2003b; Baranska et al., 2006; Solovchenko et al., 2006). By means of calibrations on spectral readings from 400 up to 1700 nm also quality attributes like fruit firmness, aroma, acid content and SSC in apples (Lammertyn et al., 1998; Zude et al., 2006), mango (Schmilovitch et al., 2000), cherry (Carlini et al., 2000) and citrus fruit (Miller and Brown, 2004) have been non-destructively measured.

Early approaches of *in-situ* spectroscopy addressing variable contents of pigments were applied by using stationary two-filter instruments (Birth and Norris, 1965; Massie and Norris, 1975). Fiber optics were used later to separate the light source from the detector, which made the devices portable (Birth, 1967; Chen and Nattuvetty, 1980). As UV/VIS and NIR instruments are equipped with miniaturised optical modules they become handier and high-resolution photodiode arrays give access to better calibration capabilities for quality-

and composition-related samplings directly on the plant (Herold et al., 2005; Zude et al., 2006). In this regard, portable spectrophotometers were introduced in practice to measure the site-specific variability of fruit SSC within an orchard, caused by deficiencies in water and nutrition supply (Peiris et al., 1999; Zude et al., 2008a). Since similar approaches of remote sensing from satellites were used for spatial canopy analysis (Rouse et al., 1974; Johnson et al., 2001; Hall et al., 2003; Perry et al., 2010), the fruit quality assessment on single trees potentially has deep impact for a geo-referenced harvest management (Zude et al., 2008a). Recently, for detecting properties which are variable within a tree or more specifically within one and the same fruit, hyperspectral imaging integrates the main features of non-destructive spectroscopy into spatial information from the product. This makes quality assessments of fruit and vegetables more feasible, inspecting insufficient nutrition and water supplies for single plants as well as defects and patchy ripeness of single crops (Lu, 2007; Nicolai et al., 2007; Qin et al., 2013).

However, the spectral sum signal of non-destructive readings is principally affected by diffuse light distribution in biological materials. This leads to perturbations through coinciding absorption as well as scattering depending on the phenotype of the sample and varying seasonal effects (Allen, 1964; Butler, 1964; Birth, 1978; Buschmann and Nagel, 1993). Consequently, the robustness of calibrations on non-destructive data is decreasing which causes the need for model re-calibration (Peirs et al., 2003; Golic and Walsh, 2006; Zude et al., 2011).

2.1.2. Coinciding absorbance

Spectral readings generally provide a high sensitivity of measurement regarding variable amounts of pigments in vegetables and fruit (Birth et al., 1957; Butler, 1964). In comparison with methods that only acquire shifts in colour (CIELAB, RGB etc.), spectrophotometric techniques are used to record wavelength-specific light absorption of individual pigments (Mackinney and Chichester, 1954). Thus, changes in chlorophylls, carotenes and xanthophylls can be non-destructively observed while they are still accumulated in thylakoid membranes of higher plant chromoplasts. Also pigment-protein-complexes remain untouched where solar energy is transferred into functional photosynthetic reaction centres (Hirschberg, 2001). Variable amounts of anthocyanins can be estimated, which are located in vacuoles, with a function of protecting against chlorophyll bleaching in intense light.

However, in transparent solutions the concentration of the light absorber is linearly correlated with optical extinction and can be determined by means of a specific absorption coefficient. The ratio of wavelength-dependent absorption intensity is thereby directly proportional to the concentration of the absorber (Lambert-Beer's Law). In contrast, biological tissues show diffuse light reflection or transmission through covering and multiple scattering events in all green, yellow and red parts of leaves, vegetables and fruit. This leads to an increased light absorption, which is not only caused by light extinction (Lancaster

et al., 1994). Additionally, due to their coinciding spectral profiles, changes in amounts of chlorophylls, carotenoids and anthocyanidins result in an additive spectral signal, not strictly apparent in colour gradients (Figure 2.1).

Beyond that, the light absorption of chlorophylls in green leaves and immature fruit have strong spectral masking effects that make other compounds with minor absorption intensity invisible to instruments like colorimeters that only record parts of the whole VIS spectrum. Masking effects are always due to highly overlapping light absorption by different pigments in a mixture. It is consequently difficult to separate such coincided signals into the specific spectral signatures of each individual pigment involved.

2.1.3. Scattering

When non-destructive spectroscopy in reflectance or transmittance mode is applied on intact fruit or vegetables, the spread of incident light from UV/VIS and NIR wavelengths is always characterised by variable scattering coefficients (μ_s), which complicates quantitative analyses of chromophoric compounds. Nevertheless the variation of μ_s within the fruit maturity has been used in practice to predict the ripeness of apples (Lu, 2003; Qing et al., 2008b; Cen et al., 2013) kiwi fruit (McGlone and Jordan, 2000; Baranyai and Zude, 2009) and bananas (Hashim et al., 2013).

However, during the traveling of photons through biological tissue, numerous reversible events of light refraction occur, due to optical inhomogeneities of different sized particles and shapes of cell organelles as nucleus, mitochondria, membranes or lipid and protein complexes (Birth, 1978; Cheong et al., 1990). Consequently, different photon propagation can be observed (Figure 2.2) and measured either by means of backward scattered light (diffuse reflection) off the surface or by forward scattering (diffuse transmittance) through the sample (Mackinney and Chichester, 1954; Birth et al., 1957). In this regard the length of photons' travelling paths depends on the properties of the medium penetrated and can be described by distribution models using Mie or Rayleigh scattering or the Kubelka-Munk theory (Fukshansky et al., 1993; Leonardi and Burns, 1999). Generally, μ_s is wavelength-dependent in turbid plant material, exponentially increasing at short VIS and UV wavelength ranges (Birth, 1978; Martens et al., 2003). According to pigment analyses on living products, this gives inadequate information about the actual distribution of photons and the plausibility of energy transfer in a certain absorber (Krivoshiev et al., 2000; Martens et al., 2003). Thus variable fruit properties and variations within the growing season affect the calibration of models for quality prediction, and consequently the correlation between measured physical properties and physiological attributes of samples (Zude et al., 2011). The approximate effective pathlength (L) in biological tissues can be determined by the reduced scattering coefficient (μ_s'), which is the reciprocal value of L (Tuchin, 1997; Cubeddu et al., 1999; Zude et al., 2011).

However, since the spectral readings of light after diffuse reflection only provide informa-

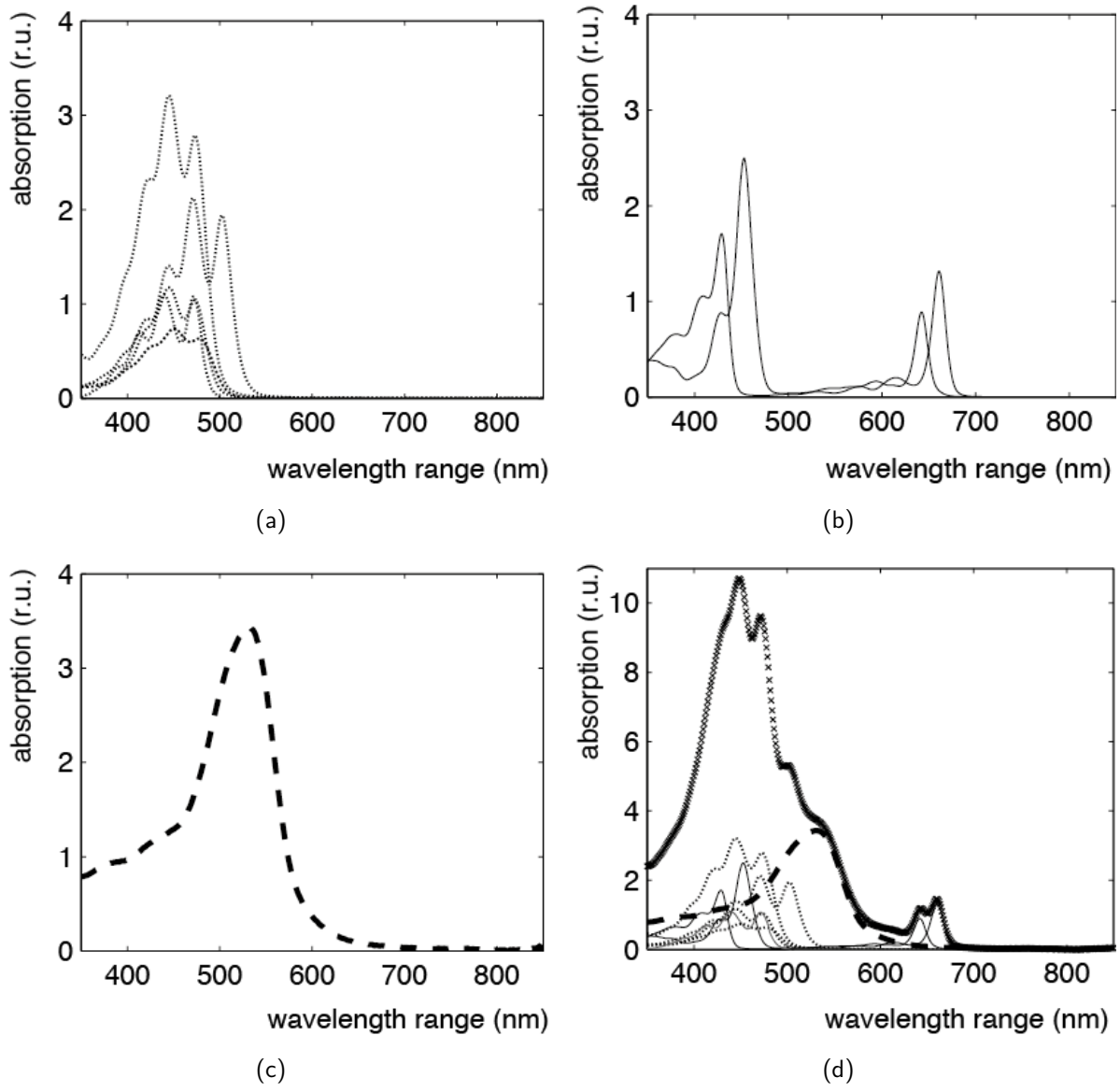


Figure 2.1.: Spectral masking effects through coincided spectral absorption of (a) individual carotenoids (dotted), (b) chlorophylls (thin solid) and (c) anthocyanidins (dashed). (d) In a mixture of individual pigments a sum signal (crosses) is measured.

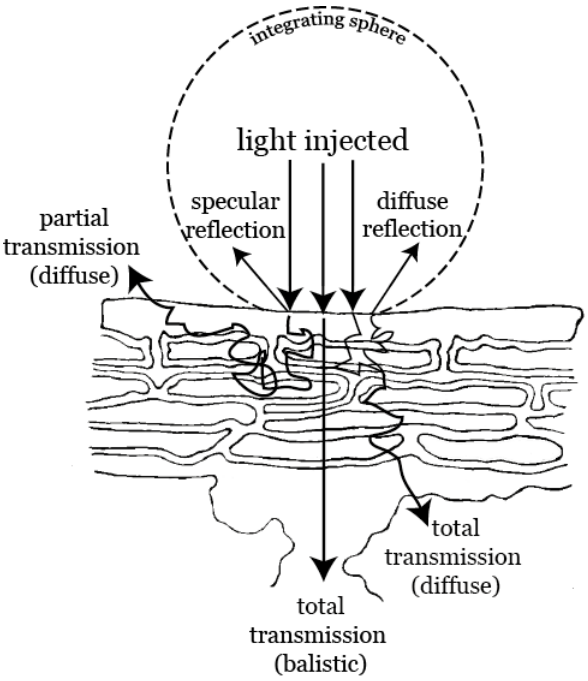


Figure 2.2.: Radiative transfer and photon transport in biological tissue (modified from Birth et al., 1957 and Abbott, 1999)

tion on constituents from the upper cell layer, measurements in transmittance mode offer data from the lateral volume of the sample. But this geometry of measurement is restrictively adaptable for samples of low optical density such as tomatoes, grapes or thin leaves (Birth et al., 1957). In this regard, spectral readings applied on optically dense apple fruit showed very low transmittance intensities and high dependencies on the cultivar (Blanke and Notton, 1992). Moreover, certain cell structures like the apple core or the cherry stone which are impermeable for light from UV/VIS wavelengths were detected as extinct. This could provide higher absorption intensities and lead to erratic results in terms of the analysis of chromophoric compounds. In contrast, in partial transmittance geometry the volume of turbid plant medium travelled by photons is shown to be relatively independent on environmental conditions and fruit properties (Zude and Herold, 2002; Herold et al., 2005).

In terms of pigment analyses, the variation of μ_s has a significant impact on the spectral profiles of absorbers. Since spectral readings in the UV part are pertubated on one side by strongly coinciding absorption of numerous carotenoids, in samples additionally showing high values of μ_s, the signal of non-destructive measurement is the sum of complex interaction between coinciding absorption and variable scattering coefficients. In this regard preprocessing approaches have been tested for the correction of non-destructive recorded VIS/NIR spectra (Isaksson and Naes, 1988; Naes et al., 1990; Dhanoa et al., 1994; Helland et al., 1995; Wold et al., 1998; Qing et al., 2008a).

2.2. Spectral indices

A practical and robust method for analysing non-destructive spectral readings according to variable amounts of chromophors is the calculation of vegetation indices (Table A.3). Primarily conceived to analyse optical remote sensing data from satellites and airborne systems (Rouse et al., 1974), the concept of spectral vegetation indices has been frequently tested in near distance spectroscopy since then (Gitelson and Merzlyak, 1996; Lichtenthaler et al., 1996). This has resulted in the conclusion that varying contents of chlorophylls and CAR_{total} are suitable indices, detectable by instruments and are well correlated to fruit maturity and quality (Olsen et al., 1967; Merzlyak et al., 1999).

The principle of index development is, in general, that of selecting such wavelength ranges of reflectance or partial transmittance, whose coefficients of variation highly correlate with variable plant properties. Valuable information about chromophoric compounds can then be gathered by estimating the positions of the absorption maxima or, more precisely, the signature of a whole spectrum (Britton, 1993). Characteristic ranges of UV/VIS light absorption in biological tissues that correspond to chlorophylls, carotenoids or anthocyanidins are shown in Figure 2.3 (grey coloured wavelength bands). Spectral variations in the VIS range have been found between 660-700 nm and 530-550 nm respectively and are suggested to be sensitive to changes in the CHLa content of photosynthetically active organs (Gitelson and Merzlyak, 1994; Gitelson and Merzlyak, 1996; Lichtenthaler et al., 1996). Reflectance in the near UV and VIS range (300-500 nm) is essentially dependent on the varying contents of carotenes and xanthophylls (Hilbert and Jansen, 1934; Miller et al., 1935; Zechmeister and Polgar, 1943; Zscheile and Porter, 1947; Hager, 1970), but simultaneously masked by short-wave chlorophyll absorption and highly-increased scattering effects (Pflanz and Zude, 2008; Zude et al., 2011). At last, spectral indices are computed as ratios, differences or linear combinations of (partially) transmitted or reflected light in the UV/VIS and NIR wavelength ranges. They provide a simple method for describing spectral variations caused by different pigment contents in leaves and fruit.

The commonly applied Normalised Difference Vegetation Index (NDVI) is a ratio that corresponds to the minimum light reflectance due to CHLa absorption at 680 nm and a high reflectance in the NIR range. Above 750 up to 800 nm, light reflectance is in principal essentially influenced by scattering and less by absorption effects of chromophors or water. Consequently the NIR reference is well-suited to the minimisation of scatter effects. However, due to its lower sensitivity to higher levels of chlorophyll, the NDVI was emphasised to not be suitable for chlorophyll determination in leaves (Lichtenthaler et al., 1996). Nevertheless in non-destructive spectroscopy applications using partial light transmission the NDVI was shown (with known limits) as acceptable indicator for fruit maturity, due to the high variability of CHLa contents during fruit's maturity (Zude, 2003; Kuckenberg et al., 2008).

With an in-depth knowledge of chromophore-sensitive spectral variation, several approaches have been made to develop more accurate reflectance indices by including additional wave-

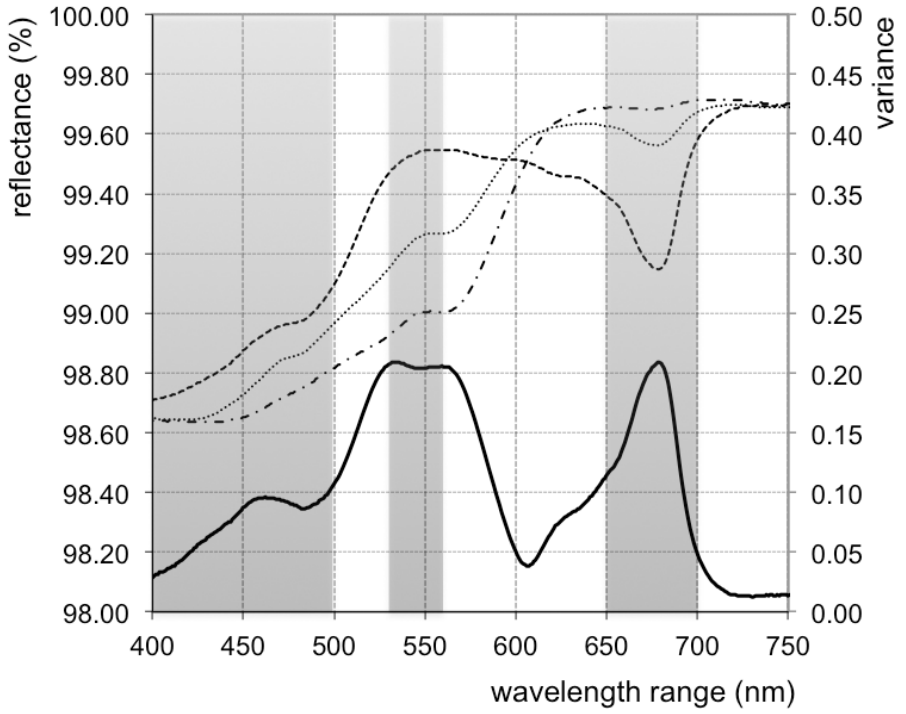


Figure 2.3.: Non-destructive UV/VIS recordings of ripening tomato fruit. Unripe (dashed), intermediate ripe (dotted) and full ripe fruit (dashed-dotted). The variance (thick solid) results from intensity differences between spectral readings of unripe and fully ripe fruits. Grey coloured areas show such wavelength ranges which have the highest spectral variance. Data from Paper I (Pflanz and Zude, 2008).

length ranges (Buschmann and Nagel, 1993; Gitelson and Merzlyak, 1994; Merzlyak et al., 1999; Richardson et al., 2002; Zude, 2003; Solovchenko et al., 2005). In this context, a series of linear equations have been shown to be suitable for the estimation of changes in chlorophyll, carotenoids and anthocyanidins in fruit and leaves (Table A.3). For the estimation of maturity-related changes of CAR_{total} in carrots, a carotenoid index was successfully applied (Zude et al., 2007). Through the use of the Normalised Anthocyanin Index (NAI – Table A.3) varying contents of anthocyanins in cherries and apples have also been determined non-destructively (Kuckenberg et al., 2008; Zude et al., 2011). However, poor correlations have been found in terms of carotenoids analyses by means of non-destructive readings from upper parenchyma layers of apples rich in red pigments (anthocyanins). This issue is only partially caused by the sensitivity of measurements. There is more evidence that anthocyanins have a high level of spectral masking that leads to coinciding absorption in the mid-visible (500-600 nm) wavelength range (Solovchenko et al., 2010).

In a more advanced method, the maturity-related chlorophyll degradation in fruit (see chapter 1.3.1) was determined to be well correlated with the shift of the inflection point (IP) on the longwave bound (red-edge) of CHLa absorption around 700 nm (Gitelson and Merzlyak, 1996; Zude-Sasse et al., 2002; Herold et al., 2005). Different methods of estimating this wavelength dependent position have been reviewed by Dawson (1998). The IP position was further shown to be sensitive to spectral variations in readings of light reflectance and partial transmittance (Figure 2.2) as well (Herold et al., 2005). In contrast to indices that are calculated through the use of reflectance intensities of static wavelength ranges, the IP of the red CHLa edge is also sensitive to spectral variations by bathochromic shift. This shift is, in turn, both maturity-related and attributed to pigment changes in living tissues and organic solvents as well (Fukshansky et al., 1993).

2.3. Advanced spectral analysis of single chromophors: iMLR

A successful implementation of optical technologies for measurements of biological material requires the combination of powerful sensors with suitable algorithms to detect relationships between physical or chemical properties and the quality attributes of horticultural products. Some approaches to spectral analysis have been shown in chapter 2.2, where limits are encountered in terms of individual pigment changes in living crops. However, the main issue in non-destructive optical recordings is the ability to get reliable information from scattered and coinciding spectral signals. A detection of individual carotenes and xanthophylls is particularly difficult due to simultaneous and consequently overlapping light absorption of carotenoids and chlorophylls during highly-increased scattering in the UV range.

For this correction of coincided absorption, several statistical enhancements have been developed, which provide extended information from experimental data in quantitative spec-

troscopy analysis (Thomas and Haaland, 1990). Derivative techniques (O'Haver and Green, 1976; Salinas et al., 1990; Nevado et al., 1995) and multivariate analyses (Arends et al., 1964; Herschberg, 1964; Beebe and Kowalski, 1987; Haaland and Thomas, 1988) have been compared, which are appropriate for the reduction of errors of calculation due to coinciding absorption. Nowadays, such methods have been successfully adapted to determine mixtures of various chemical compounds in horticultural crops when applying UV/VIS spectrophotometry (Marr et al., 1995; Ni and Gong, 1997; Zude and Herold, 2002; Zude, 2003; Polder et al., 2004; Merzlyak, 2006; Pflanz et al., 2010). In terms of pre-processing approaches, multiplicative signal correction (MSC) are being used to separate chemical light absorbance and physical light scatter effects in highly-scattered turbid media (Martens et al., 2003). Moreover, a time-resolved measurement of light distribution in biological tissue and dense suspensions may help to solve scatter problems in non-destructive estimations of plant physiology (Fukshansky et al., 1993; Zude et al., 2011). However, the major disadvantage of derivative spectroscopy for implementation in *in-situ* spectroscopy is the decreasing signal-to-noise ratio as the order of the derivative is increasing (Cameron and Moffatt, 1987). In particular this occurs at short wavelengths, which are the main spectral absorption range of carotenoids. Here the signal of reflectance decreases due to rapid increasing scatter effects, or due to the intensity of light source, which is partially low by design at short wavelength ranges. Further, multivariate statistics were shown to be suitable for quality prediction over a wide range of horticultural crop attributes (Norris, 1983; Thomas and Haaland, 1990). Methods such as principal components analysis (PCA) or partial least square regression (PLS) are being used for the reduction of the multidimensionality of spectral data and bringing out the relationships between measurements and attributes (Kress and Brimelow, 2001). Nevertheless, only a few PLS approaches have been used for the multivariate prediction of chromophoric compounds (Zude et al., 2011). With regards to changing seasonal conditions or new variety samples, PLS calibrations may generate models of less accuracy, which would result in high errors, low regression and high bias (Janik et al., 2007). PLS calibration may also tend toward high relative errors in the prediction of low pigment contents.

Nevertheless, methods of simple equation systems discussed and applied by MacKinney (1941), Zscheile and Porter (1947) and Arnon (1949), finally reviewed and improved by Wellburn (1994), Lichtenthaler and Buschmann (2001) and Porra (2002) are being used today for spectral analysis of fruit and vegetables containing mixtures of CHLa, CHLb and carotenoids. Extended as MLR, these approaches are independent from calibration procedures and thus robust against seasonal and variety-related variability (Pflanz and Zude, 2008). However, MLR methods are reasonable only in a certain (Lambert-Beer valid) domain of spectrophotometric analysis. It requires linear responses with no interfering signals from interactions of constituents, low noise and no collinearities (Beebe and Kowalski, 1987).

A spectral separation of individual chlorophylls, carotenes and xanthophyll is being applied in the present work through an iterative MLR approach. The principle of stepwise fitting of known spectral signatures on measured and coincided sum signals is shown in Figure 2.4a-d. For this purpose, adjusted pigment compositions were built from laboratory standards and used to determine their spectral characteristics in simulated natural mixtures (Pflanz and

Zude, 2008). It is assumed, that between 600 and 700 nm exclusively CHLa and CHLb absorb light from VIS range. Through this, the fitting in the range of main chlorophyll absorption with a low influence of scatter effects from the biological tissue, is of high accuracy. Due to the second short wave absorption of chlorophylls, the strong coincided range between 350 and 500 nm can be corrected by the absorption intensities as well (Figure 2.4b). A small absorption band of LYC between 500 and 505 nm allows these steps to be iterated for other carotenes and xanthophylls as well (Figure 2.4c and d).

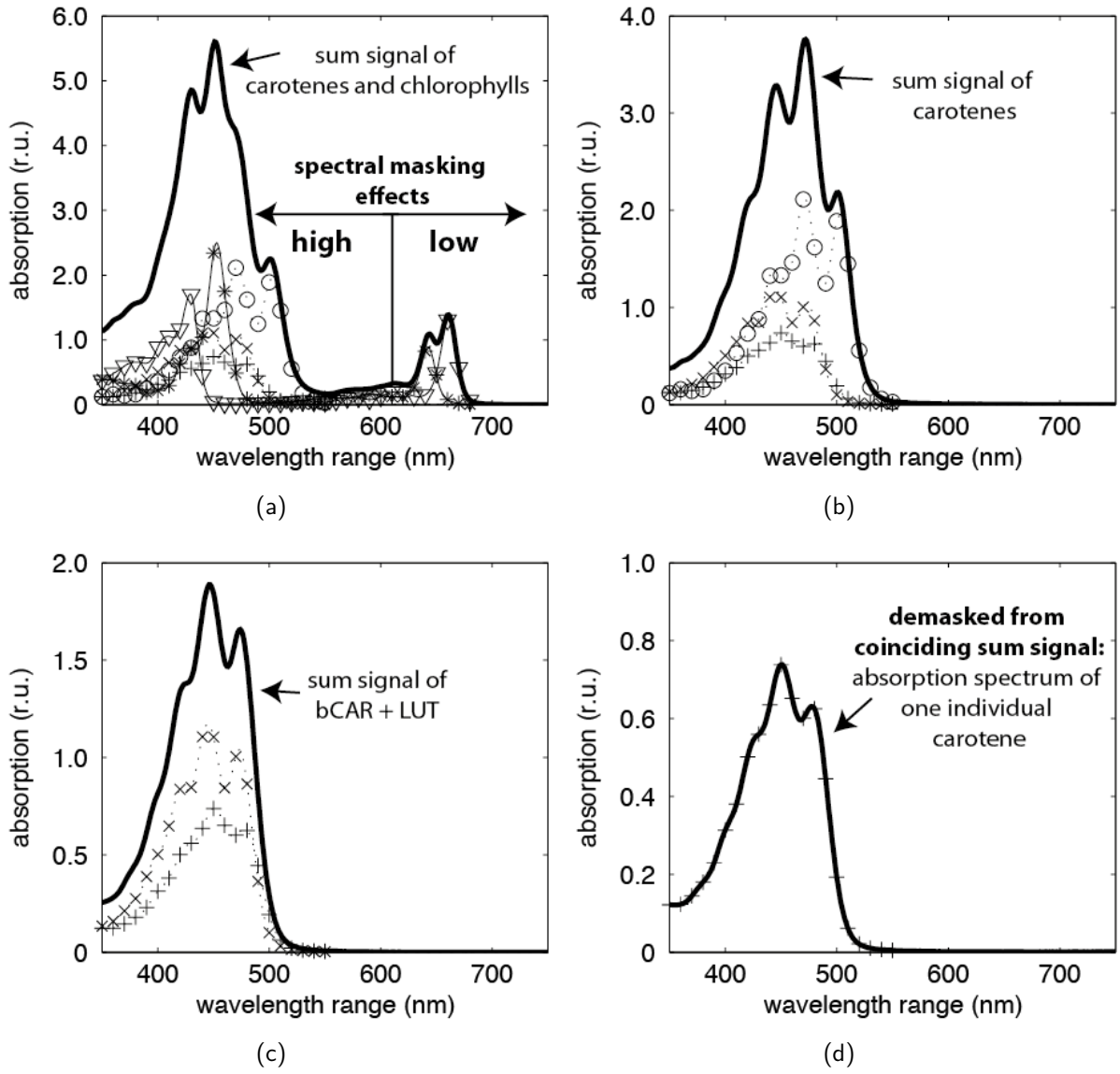


Figure 2.4.: Principle of iMLR by taking the example of bCAR separation from a mixture of carotenes and chlorophylls (steps a-d). The method iteratively damasks coinciding absorption in pigment mixtures beginning at wavelength ranges of low scattering effect (red VIS range). Through the use of spectral profiles of relevant chromophors like CHLa (downward-pointing triangle), CHLb (asterisk), LYC (circle), LUT (cross) and bCAR (plus sign) each individual pigment absorption can be separated from measured sum signal. Applied here on spectral recordings of tomato extracts (data from Paper I).

3. Correction for perturbations due to coinciding absorption and scattering variation (Peer reviewed papers)

3.1. Coinciding absorption

3.1.1. Corresponding paper

Chapter 3.1 is consistent with the following publication (paper I):

Pflanz, M. and Zude, M. (2008). Spectrophotometric analyses of chlorophyll and single carotenoids during fruit development of tomato (solanum lycopersicum l.) by means of iterative multiple linear regression analysis. Applied Optics, 47(32):5961–5970.

Status: Received 3 June 2008; revised 24 September 2008; accepted 24 September 2008; posted 26 September 2008 (Doc. ID 96625); published 3 November 2008

3.1.2. Abstract

When using spectrophotometric transmittance readings of fruit extracts, the analysis of single carotenoids is difficult because of coinciding absorption bands of the various carotenoids and chlorophylls present in the solution. Aimed at the separate analyses of pigments, an iteratively applied linear regression was developed based on spectral profiles of pigment standards. The iterative approach was validated by dilution series of pigments and compared with commonly applied equation systems. High coefficients of determination and low measuring uncertainties were found for CHLa and CHLb ($r^2 \geq 0.99$, root mean square error $rmse \leq 10\,\%$). Carotenoids were separately analysed with $r^2 = 0.99$, $r^2 = 0.96$, and $r^2 = 0.98$ for LYC, bCAR, and LUT, respectively. The approach based on the spectral profiles provided low measuring uncertainties even if LUT was additionally present in the solutions, which was not possible with common data analyses. Subjecting tomato tissues to the iterative approach, contents of *in-vivo* measured pigments were calculated with $r^2 = 0.82$, $r^2 =$

0.84, $r^2 = 0.67$, and $r^2 = 0.03$ for CHLa and CHLb, LYC, and bCAR, respectively.

3.1.3. Introduction

Recently, secondary plant compounds moved into the field of interest of science and quality management because of their nutritional value in human diets. An important role has been attributed to the carotenoids, particular LYC, bCAR, and LUT, which can be found in human plasma and tissues in relation to, e.g., tomato consumption (Oshima et al., 1996). In vitro studies of plant material evidenced that these compounds are effective singlet oxygen quenchers (Di Mascio et al., 1989) and can act as free radical scavengers (Stahl and Sies, 1996; Gerster, 1997). Epidemiological studies have shown that consumption of fruit and vegetables rich in bCAR is correlated with a reduced risk of some types of cancer (Clinton et al., 1996; Fernandez et al., 1997). Studies of cardiovascular disease point to an interaction between the number of medical risks and the consumption of fruit and vegetables (Krinsky, 1998). bCAR is of particular importance, since it possesses the highest provitamin A activity, while LYC has high antioxidative capacity, and LUT is the major carotenoid in the human lens. However, health or preventive effects of isolated carotenoids could not be proved (Michaud et al., 1998; Pelz et al., 1998). The contents and compositions of pigments in tomato depend essentially on cultivation and postharvest conditions, and, even more pronouncedly, on cultivar and maturity stage (Shewfelt et al., 1988; Giovanelli et al., 1999; Shewfelt, 1999; Toor and Savage, 2006). Studies on tomato quality, however, focused mainly on homogeneity, colour, and shelf life, while more sensitive data based on single carotenoids contents were rarely taken into account. Reasons are time consuming and expensive destructive chromatographic analysis, while more feasible spectrophotometric methods for measuring on plant extracts are capable of analysing chlorophyll. But only a few methods are described for distinguishing between single carotenoids (Polder et al., 2004).

In addition to the demand for efficiency, a potential need appears in food production and processing for real-time monitoring of maturity and nutritional quality. As a result, a non-invasive approach represents the final target. UV/VIS spectrophotometry applied on fruit extracts provides an established method for measuring fruit chlorophyll and might be applied to *in-vivo* readings with minimal or no sample preparation. Non-invasive NIR spectrophotometric methods for monitoring the SSC and maturity of horticultural products have been developed in recent years (Chen and Nattuvetty, 1980; Baranska et al., 2006) and are already applied in commercial instruments (Miller and Brown, 2004; Zude et al., 2008a). Chlorophylls and carotenoids are light-absorbing pigments that can be measured by different methods in practice. Using colour cards for measuring the skin colour is inexpensive and non-invasive, but erratic and subjective. With spectrophotometry applied in the VIS wavelength range, the content of pigments is highly correlated with the absorption at the specific wavelengths. The content of chlorophylls and carotenoids provides a sensitive indicator for maturity and senescence in many fruits including tomato (Kozukue and Friedman, 2003; Polder et al., 2004). By means of sensors for monitoring the pigment content, the optimum harvest date

with maximum fruit quality can be determined in practice (Zude et al., 2006).

However, the determination of individual pigments of extracts in the laboratory and further by non-invasive spectrophotometry *in-vivo* is difficult owing to variation in the scattering characteristics of fruit tissue. Baseline correction and data preprocessing, such as multiple scatter correction, are feasible methods for diminishing the effects on the measured signals. Furthermore, the pigments are always presented in mixtures that result in coinciding absorption bands of CHLa and CHLb, as well as carotenoids.

In recent studies, methods for analyzing *in-vivo* measured spectra were proposed: the IP of the long-wave flank of the chlorophyll absorption peak (red-edge) and other indices are investigated for analyzing the content of chlorophylls (Merzlyak et al., 2003a; Zude, 2003; Gitelson et al., 2006) and carotenoids (Ni and Gong, 1997) in fruit tissues. Chemometric data processing methods such as PCA and PLS regression analysis based on multivariate variation of spectral intensities were tested and have been applied for multicomponent analysis of pigment mixtures by UV/VIS absorption spectrophotometry in food analyses (Arnon, 1949; Ni and Gong, 1997; Zude, 2003) as well as medical applications (Wu et al., 2000; Meinke et al., 2005). Such calibrations, however, are valid as long as the calibration data set captures all possible compositions occurring in the prediction data set. The influence of coinciding absorption coefficients of varying pigment compositions in the carotenoids absorbance bands cannot be solved by the available measuring protocols.

In the present work, an approach was developed for calculating CHLa and b, LYC, bCAR, and LUT separately by means of iterative MLR with respect to the specific absorption coefficients (absorption profiles) of pigment standards. The method developed was validated with dilution series of different pigment standards and fruit extracts aiming at an improved laboratory method for pigment analyses in fruit extracts. Furthermore, first attempts were carried out on different tomato tissues and maturity stages for non-invasive readings.

3.1.4. Theory

Quantitative spectrophotometry in the VIS wavelength range can be used to determine contents of pigments according to the Lambert-Beer law, which describes the relationship between the absorbance (A), the specific molar extinction coefficient (ϵ), the pathlength (l), and the absorber concentration (c). In highly diluted solutions with no influences by scattering or additional absorbers, the extinction is equivalent to the absorption (A)

$$A = \epsilon l c. \tag{3.1}$$

By using specific absorption coefficients (k) the absorbance is related to concentrations in milligrams per liter. If l=1 such as in a standard cuvette, equation 3.2 follows

$$A = kc. \tag{3.2}$$

As a result, spectrophotometry in the UV/VIS wavelength range can be used for determining colourants, but their prior chemical separation is necessary due the disturbing influence of coinciding spectral absorption intensities of varying pigments. Wet chemical separation is usually time consuming and sometimes unsuccessful. To avoid these procedures, spectral analysis using various chemometric methods has been used (Arnon, 1949; Ni and Gong, 1997; Wu et al., 2000), but varying pigment compositions may disturb the calibration models. For measuring on the sum spectrum of CHLa and CHLb in plant extracts different approaches were proposed to distinguish between the pigments in a varying mixture. The most often used method to determine individual concentrations of mixed pigments in plant extracts was developed by Arnon (1949). It is based on the spectrophotometric measurement of the extinction in the red wavelength region at two absorption maxima for CHLa and CHLb.

Based on the Lambert-Beer law Arnon (1949) developed a linear system of equations with determinants representing the specific extinction coefficients (k) obtained first by MacKinney (1941) followed by other working groups (Table 3.1). With the principle of Arnon (1949) the sum extinction of mixed extracts at two different wavelengths (1, 2) was described by an equation system (Equation 3.3)

$$\begin{aligned} A_1(\lambda) &= k_{11}c_1 + k_{12}c_2 \\ A_2(\lambda) &= k_{21}c_1 + k_{22}c_2. \end{aligned} \tag{3.3}$$

By resolving the equation system for c_1 and c_2 (concentration of CHLa and b, respectively) using the determined specific extinction coefficients (k_i) of the pigment under question concentration can be calculated (Equation 3.4)

$$\begin{aligned} c\text{CHLa} &= 12.70A(663) - 2.69A(645) \\ c\text{CHLb} &= 22.90A(645) - 4.68A(663). \end{aligned} \tag{3.4}$$

cCHLa and cCHLb represent the concentration of CHLa and CHLb calculated in μg mL^{-1}.

Although the obtained extinction coefficients were based on inaccurate spectrophotometric data regarding the poor resolution of spectrophotometers in the 1940s and acetone solvents had been mixed with water (Wellburn, 1994), the employed simultaneous equations were used over half a century by many researchers and in most laboratories dealing with fruit and vegetables. In recent publications the determination of accurate extinction coefficients for CHLa and b in buffered 80 % aqueous acetone was reported (Porra et al., 1989). High

Table 3.1.: Specific extinction coefficients (k) of CHLa and CHLb estimated by different working groups

			k (l g^{-1} cm^{-1})		
λ (nm)	Resolution (nm)	Solvent	CHLa	CHLb	Reference
663	1-4	80 % acetone	82.04	9.27	(Mackinney, 1941)
645			16.75	45.60	
663.6	0.1-0.5	80 % acetone	85.95	10.78	(Porra et al., 1989)
645			20.79	51.84	
660.8	0.1-0.5	diethyl ether	100.90	5.98[a]	(Porra et al., 1989)
642.6			15.00[a]	62.00	
660	1-4	diethyl ether	102.00	4.50	(Comar and Zscheile, 1942)
642			16.30	57.50	
662	1-4	diethyl ether	100.90	4.75	(Wellburn, 1994)
644			19.37	62.00	

[a] Recalculated specific extinction coefficients based on equation systems of Wellburn (1994).

purified CHLa and CHLb were measured and used to estimate coefficients without variable micro-contamination by chlorophylls degradation solvents (Table 3.1).

Using these specific extinction coefficients of Porra and co-workers (Porra et al., 1989) improved equation systems became available (Equation 3.5)

$$\begin{aligned} c\text{CHLa} &= 12.725A(663.6) - 2.55A(646.6) \\ c\text{CHLb} &= 20.31A(646.6) - 4.91A(663.6). \end{aligned} \tag{3.5}$$

Equations for diethyl ether solvent compositions were determined (Equation 3.6), which also included coefficients enabling CAR_{total} analysis by measuring the absorption at 470 nm (Wellburn, 1994)

$$\begin{aligned} c\text{CHLa} &= 10.05A(662) - 0.77A(644) \\ c\text{CHLb} &= 16.37A(644) - 3.14A(662) \\ c\text{CAR}_{total} &= (1000A(470) - 1.28c\text{CHLa} - 56.7c\text{CHLb})/205. \end{aligned} \tag{3.6}$$

To determine the LYC content of tomato tissue, Fish and co-workers (Fish et al., 2002) used the extinction coefficient of $k = 31.2$ and the absorbance value at 503 nm versus a blank of a hexane solvent. The absorbance peak at 503 nm was used to minimize interference from other carotenoids. Also, the influence of chlorophyll absorption is low at this wavelength

$$c\text{LYC} = 31.2A(503). \tag{3.7}$$

In conclusion, according to the approach of Arnon (Equation 3.3) an equation system can be built for the separated quantitative analysis of a solution consisting of several pigments. Linear equation systems can be computed, if the number of equations is equal to the number of absorber concentrations needed (c_n)

$$\begin{aligned} A_1(\lambda) &= k_{11}c_1 + k_{12}c_2 + \cdots k_{1n}c_n \\ A_2(\lambda) &= k_{21}c_1 + k_{22}c_2 + \cdots k_{2n}c_n \\ &\vdots \\ A_n(\lambda) &= k_{n1}c_1 + k_{n2}c_2 + \cdots k_{nn}c_n. \end{aligned} \tag{3.8}$$

While overlapping absorption by other, e.g., tomato, pigments in the wavelength range of LYC absorption maximum at 503 nm is low, the measured sum spectra of bCAR absorption between 400 and 500 nm is affected strongly by coinciding absorption of different carotenoids and shortwave absorption of chlorophylls. The equation system must be calculated exactly to be able to determine the concentration of the carotenoids LYC, LUT, and bCAR, taking into account the shortwave absorption of CHLa and b in the mixture.

The method adapted to the question of spectral quantitative analysis is to create a calibration equation system, which uses the known wavelength-dependent absorption intensities of single compounds. In the present study, a set of standard solutions are produced that represents the possible components of the sample. The expected range of concentrations is measured. CHLa and CHLb are measured within the ranges as they occur in plant extracts (Brown et al., 1982; Kisner et al., 1982; Marr et al., 1995). The unknown samples will be measured with the same experimental setup, and the equations will be used to predict the concentration of calibrated components.

In the present study, an approach was carried out to calculate carotenoids of tomato fruits separately for LYC, bCAR, and LUT by using simultaneous multicomponent spectrophotometry. With a large number of data points of the absorption spectrum, overdetermined simultaneous equations were used to find the concentrations of chlorophylls and carotenoids. The relationship between concentration and absorbance is often nonlinear, because of deviation from the Lambert–Beer law (Brown et al., 1982). Within a limited range of linearity, however, the resulting curve can be approximated as linear by adding a nonzero intercept (e) to the equations. Then, for a system of multiple components, the Lambert–Beer expression can be expanded to include absorbance of each of the components at each of the analytical wavelengths (n = 400), where $A_i(\lambda)$ is the measured sum signal of sample absorbance at the nth wavelength, c_m is the concentration of the nth component, and k_nm is the wavelength-dependent coefficient

$$
\begin{aligned}
A_1(\lambda) &= k_{11}c_1 + k_{12}c_2 + \cdots k_{1m}c_m + e \\
A_2(\lambda) &= k_{21}c_1 + k_{22}c_2 + \cdots k_{2m}c_m + e \\
&\vdots \\
A_n(\lambda) &= k_{n1}c_1 + k_{n2}c_2 + \cdots k_{nm}c_m + e.
\end{aligned}
\tag{3.9}
$$

Converted to a common matrix expression, the equation system can be written as

$$
\begin{bmatrix} A_1(\lambda) \\ A_2(\lambda) \\ \vdots \\ A_n(\lambda) \end{bmatrix} = \begin{bmatrix} k_{11} & k_{12} & \cdots & k_{1m} \\ k_{21} & k_{22} & \cdots & k_{2m} \\ \vdots & & & \\ k_{n1} & k_{n2} & \cdots & k_{nm} \end{bmatrix} \cdot \begin{bmatrix} c_1 \\ c_2 \\ \vdots \\ c_n \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \\ \vdots \\ e_n \end{bmatrix} \text{ or } \quad A = KC + e. \tag{3.10}
$$

If the wavelength-dependent resolution of measurements is higher than the number of components in the mixture, the system is overdetermined. This consequently has no exact solution but gives a least squares fit, where the main goal is to minimize the error (e) of regression. This objective is reached if the squared sum of regression residuals is as low as possible,

$$
\sum_{i=1}^{n} e_i^2 = \sum [A_i(\lambda) - (k_{i1}c_1 + k_{i2}c_2 + \cdots k_{im}c_m)]^2 \rightarrow min. \tag{3.11}
$$

The matrix K can be multiplied by its transpose K^T to get a square matrix with m components (Marr et al., 1995),

$$
K^T A = K^T K C. \tag{3.12}
$$

If the columns of K are linearly independent (the matrix has full rank), $K^T K$ is an invertible matrix, and the solution of equation 3.11 is given as (Marr et al., 1995)

$$
C = (K^T K)^{-1} K^T A. \tag{3.13}
$$

Once the K matrix is calculated, its inverse can be used to determine the concentrations of unknowns from measured absorbances.

However, the *in-vivo* determination of individual pigments, e.g., in tomatoes, by means of non-invasive spectrophotometry is more difficult owing to the influence of scattering and anisotropic tissue affecting the apparent absorbance of photons. Recently, the analysis of *in-vivo* measured spectrophotometric data was approached by means of PLS regression and PCA (Ni and Gong, 1997; Polder et al., 2003; Zude et al., 2008a).

In the present work the iMLR analysis was applied to calculate chlorophylls and carotenoids separately with respect to the specific absorption coefficients of pigment standards. The approximation of all pigment standards to the sum fruit spectrum was reached iteratively. In the red wavelength range the absorption of chlorophylls can be assumed to be the major source for variation. Therefore, the first approximation step is to fit the concentrations of chlorophylls to the sample spectrum in the wavelength range between 550 and 780 nm (long-wave chlorophyll peak). Thus, by means of known specific absorption coefficients of pigment standards, the shortwave intensities of absorption can be calculated. In the following step the measured sum spectrum can be corrected by the shortwave absorption of chlorophylls. In the corrected spectrum the intensities of carotenoids remain, which can now be calculated equally to the chlorophyll estimations by using overdetermined simultaneous equations based on the specific absorption coefficients obtained from standards and the C matrix derived from the calibration.

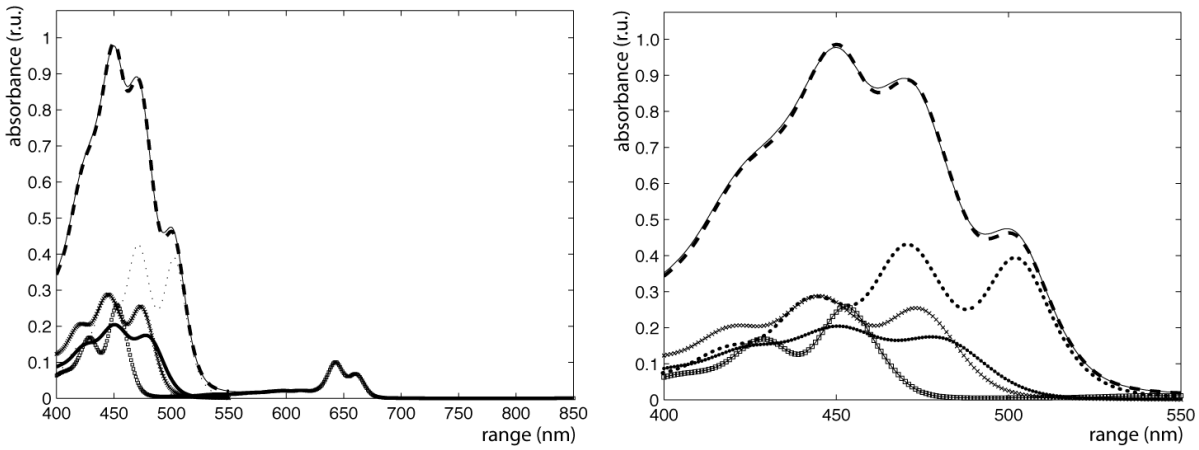
3.1.5. Materials and methods

A. Spectra Acquisition

Spectra of pigment standards (Roth, Germany) and fruit extracts were measured in transmittance mode with a double-beam scanning system (Lambda 950, PerkinElmer). The UV/VIS/NIR spectrophotometer is equipped with two monochromators in the Littrow configuration and a holographic grating with 1440 lines mm^{-1} (UV/VIS) and 360 lines mm^{-1} (NIR). A silicon-based photomultiplier tube in the UV/VIS wavelength range and a Peltier-cooled PbS detector for NIR served as detectors. The wavelength range was set to 300–1500 nm with a resolution of 1 nm. A tungsten halogen lamp was applied aslight source in the VIS and NIR range, using a slit width of 0.5 nm. At the beginning of measurements an auto-zero-correction was carried out. The spectrophotometer was controlled by commercial software (UVWinLab 5.1, PerkinElmer).

B. Software Tool for Pigment Prediction

Specific extinction coefficients of pigments were determined at $c = 0.1\,mg\,L^{-1}$ in the wavelength range λ = 400-800 nm (Table 3.2). The data for CHLa, CHLb, bCAR, LYC,



(a) (b)

Figure 3.1.: Example data set of pigments within a mixture typical occurring during the ripeness of tomato fruit. The sum spectrum of all constituents is fitted by the determination of iMLR. Fitted sum spectrum (short dashed), LYC (dotted), bCAR (thick solid), LUT (cross), chlorophylls (squares), measured sum spectrum of extract (thin solid). To clarify coinciding effects the full wavelength range (a) and the range of carotenoid absorption (b) are shown.

Table 3.2.: Calculated linear system of equations for typical tomato pigments

	Determinants				
Pigments c (mg/100mL)	A (661)	A (643)	A (503)	A (451)	A (445)
CHL a	0.765	-0.047	0.000	-0.009	0.008
CHL b	-0.172	1.144	0.000	-0.010	0.009
bCAR	2.490	-11.921	-0.147	10.858	-9.942
LYC	-0.144	0.665	0.547	-0.575	0.496
LUT	-1.176	4.801	-0.567	-5.829	6.230

and LUT were used as vectors corresponding to the specific pigment signature. To check linearity, pigment standards were measured in a dilution series ($n = 4$) of diethyl ether ($c = 0.1\text{-}1.0\,\mathrm{mg\,L^{-1}}$). These profiles were used as a database for MLR analysis to fit the recorded in vitro and *in-vivo* spectra by means of the least squares error. Iteratively, in the first step chlorophylls were fitted, while other pigments were fitted after calculating the chlorophyll absorption influence in the blue wavelength range. For this protocol, a script based on MATLAB Release 14 (Mathworks) was developed. The separated pigment profiles were calculated from the apparent sum spectrum (Figure 3.1).

C. Validation Test Set

Stock solutions ($c = 1\,\mathrm{mg\,mL^{-1}}$) of each pigment (CHLa and CHLb, bCAR, LUT, and LYC) were freshly prepared in diethyl ether. Solutions ($n = 59$) were obtained that ranged from single pigments to mixtures that represented all pigments. The concentration range

of each pigment present captured 0.05 mg/100 mL to 0.45 mg/100 mL. All samples were prepared in the laboratory at a very low level of lighting and at a constant temperature of 20°C without the influence of sunlight.

D. Non-invasive Measurements

In-vivo experiments were carried out on tomato (*Solanum lycopersicum* L.) fruits ($n = 60$) at different ripeness stages varying from immature green to intense red colour. The ripeness was graded subjectively by using a tomato colour chart (OECD, 1992) that is commonly used by breeders as well as research laboratories. A laboratory spectrophotometer (Lambda 950, PerkinElmer) was used for spectral recordings in the VIS wavelength range (350-800 nm). Diffuse reflectance on fruit surface was measured non-invasively, using an integrating sphere. Two spectra were recorded and subsequently averaged in the equatorial region on each fruit surface.

E. Chemical Analyses

Fresh tomato slices (2 mm thickness, 14 mm diameter) of each fruit from outer pericarp were homogenized (Ultra Turrax T18, IKA Works). After a wet chemical 80 % acetone/diethyl ether extraction transmittance spectra were recorded from solvents (Porra et al., 1989; Lichtenthaler et al., 1996; Fish et al., 2002). Absorption was measured in the VIS wavelength range (350-800 nm) with a resolution of 1 nm.

3.1.6. Results

A. Results of Validation on Standards

Standard methods (Nagata and Yamashita, 1992; Wellburn, 1994) and iMLR were evaluated by dilution series ($n = 59$) of pigment standards. According to the principles of Arnon (1949), Porra et al. (1989), Nagata and Yamashita (1992) and Wellburn (1994), linear equations systems were built to compare the measuring uncertainty of iMLR with common used equations (Table 3.2). Therefore, the specific extinction coefficients of each pigment standard were determined by using stock solutions in diethyl ether (Table 3.3).

As expected, a high coefficient of determination ($r^2 = 1$) of adjusted and measured pigment concentration was found with all methods for CHLa and CHLb (Table 3.4). In comparison of linear equations calculated in the present work and the formerly described method of iMLR with common methods, the relative $\%\, rmse$ was always low for chlorophylls, with values between 6.0 % and 9.3 %.

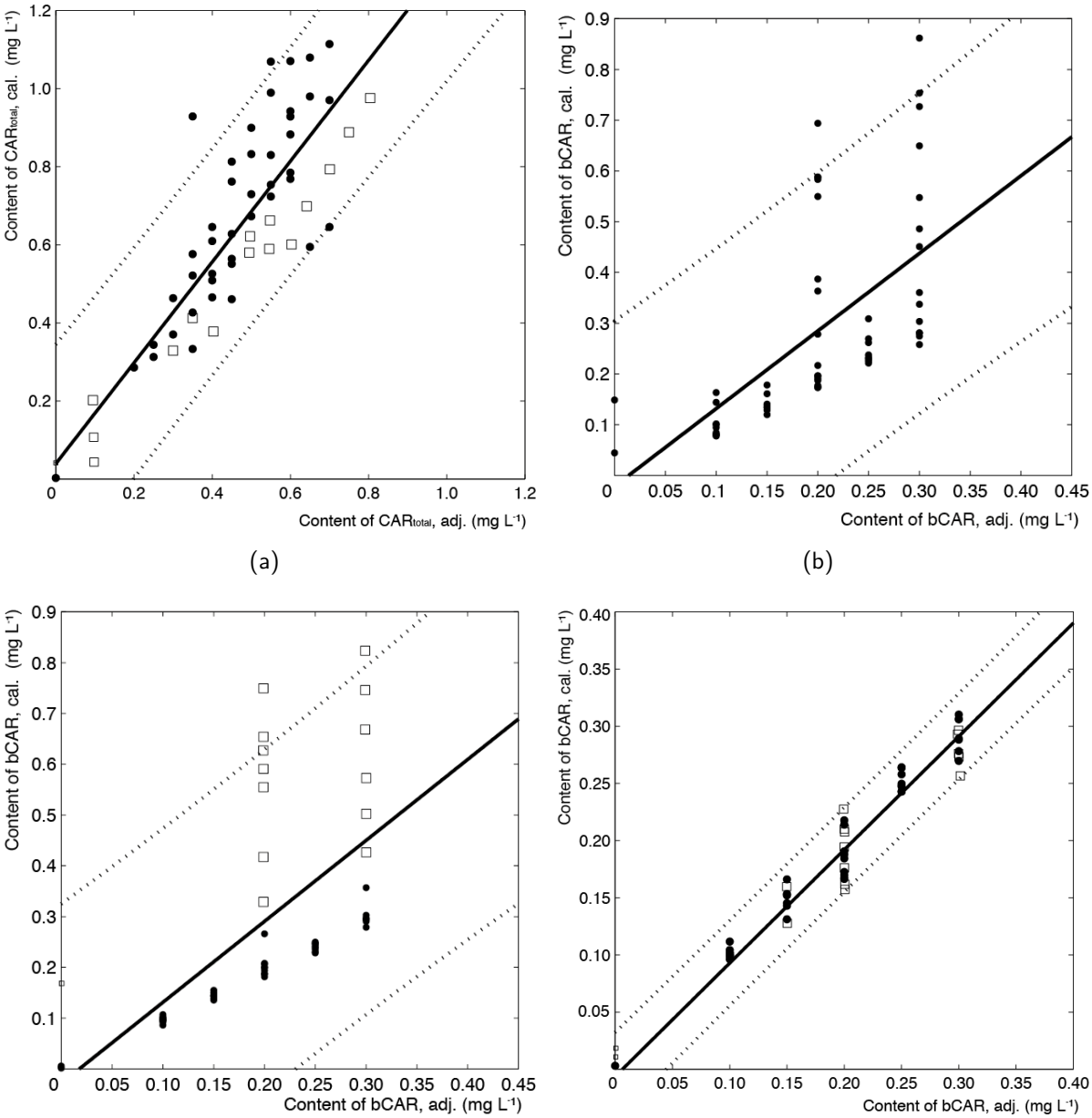


Figure 3.2.: Regression of adjusted (adj.) and calculated (cal.) contents of bCAR compared by different methods of spectral analysis: (a) Wellburn (1994), $r^2 = 0.73$, (b) Nagata and Yamashita (1992), $r^2 = 0.42$, (c) developed equation system, $r^2 = 0.84$, (d) iMLR, $r^2 = 0.96$. Open squares represent adjusted solutions that contain LUT, which can be found in early ripeness stages of tomato fruit.

Table 3.3.: Extinction coefficients of calibration standards for typical tomato pigments solved in diethyl ether

	k (100 mL mg^{-1} cm^{-1})				
Wavelength (nm)	CHLa	CHLb	bCAR	LYC	LUT
661	1.318	0.066	0.001	0.000	0.000
643	0.197	0.892	0.001	0.000	0.000
503	0.024	0.038	0.138	1.933	0.066
451	0.036	2.442	0.738	1.307	1.071
445	0.133	1.613	0.703	1.398	1.168

The content of CAR$_{total}$ could be separated by the method of Wellburn (1994) with $r^2 = 0.73$ (Figure 3.2). However, the obtained $rmse$ was high, with a value of 41.4 %. Using the equations of Nagata and Yamashita (1992) LYC calculated separately was well correlated with adjusted mixtures ($r^2 = 0.98$), while the coefficient of determination for bCAR was low ($r^2 = 0.42$). After removing mixtures containing LUT, the coefficient of determination increased ($r^2 = 0.99$). Carotenoids could also be separated precisely by linear equation systems and iMLR ($r^2 \geq 0.96$). For CAR$_{total}$ the error was low, with $rmse = 4.8$ % calculated by iMLR, even low with separation into LYC ($rmse = 5.0$ %), bCAR ($rmse = 7.4$ %). With a maximum of $rmse = 20.9$ % (LUT), the $rmse$ of iMLR was still lower than the error of conventional methods (Figure 3.2 and Table 3.4).

B. Tomato Pigments Content

Tomatoes usually have reached the light-red or red stage when they are harvested and sold in supermarkets. In the present study, tomatoes at different ripeness stages with varying composition of chlorophylls and carotenoids were subjected to the analyses for evaluating the feasibility of iMLR to monitor the development of single pigment contents under postharvest conditions.

Contents of chlorophylls were calculated by using equations from Nagata and Yamashita (1992) and Wellburn (1994), respectively, for LYC and bCAR and by using iMLR. For determination of LUT in tomatoes no equation could be found in the literature. Equations for simultaneous determination of the carrot pigments aCAR, bCAR and LYC were published earlier (Nagata, 2007). Since the spectral profiles of aCAR and LUT are nearly identical ($\epsilon =$ 2550 L mol^{-1} cm^{-1}) at the same wavelength maximum of 445 nm, it would also be possible to use this equation for determining tomato pigments. However, in contrast to carrots, tomatoes have a high content of chlorophylls in early maturity stages, so the equation mentioned can be used only with tomato samples in the red stage of ripeness. According to the principle of Nagata et al. (2007), a simultaneous equation system is presented here to calculate the tomato carotenoids LYC, bCAR, and LUT (Table 3.3). Chlorophylls are degraded during tomato ripening. The loss of chlorophylls corresponds to a loss of the green colour of the pericarp. Tomatoes at ripeness stage 4 contained 15.8 μg CHLa per 1 g fw (Wellburn, 1994), 12.8 μg g^{-1} with iMLR, and 3.1 and 3.7 μg g^{-1} CHLb, respectively (Table 3.5). The CHLa

Table 3.4.: Validation results for measurement of standard solution in diethyl ether providing various pigment concentrations typical occurring in ripening tomato fruit

Test	CHLa	CHLb	bCAR	LYC	LUT	CAR_{total}
	Lichtenthaler and Wellburn (1983)		Nagata and Yamashita (1992)			Wellburn (1994)
r^2	1.000	1.000	0.980 (0.990)[a]	0.42 (0.99)[a]	–	0.730
$rmse\ (mgL^{-1})$	0.003	0.003	0.11 (0.110)[a]	0.130 (0.150)[a]	–	0.160
$rmse$ (%)	6.910	6.390	(45.300)[a]	66.440 (79.300)[a]	–	41.350
Bias	0.000	0.000	0.090 (0.090)[a]	0.110 (0.140)[a]	–	1.190
	Recalculated linear equation systems					
r^2	1.000	1.000	0.990	0.840	0.960	–
$rmse\ (mgL^{-1})$	0.004	0.003	0.010	0.050	0.030	–
$rmse$ (%)	9.300	7.090	6.150	25.880	44.020	–
Bias	0.000	0.000	0.010	0.040	-0.010	–
	iterative multiple linear regression (iMLR)					
r^2	1.000	1.000	0.990	0.960	0.980	0.990
$rmse\ (mgL^{-1})$	0.004	0.003	0.010	0.010	0.010	0.023
$rmse$ (%)	8.880	6.370	5.030	7.380	20.910	4.770
Bias	0.000	0.000	0.010	0.010	0.000	0.020

[a] No LUT in the adjusted solvents.

content decreased by 90 % during stages 4 and 6. Chlorophylls were not detectable in stage 7 and higher ripeness stages.

The LYC content and the colour classification of the ripening stages were linearly correlated (Figure 3.3b). The content increased from 3.4 μg g^{-1} fw to 141.7 μg g^{-1} fw and from 0.6 μg g^{-1} fw to 132.3 μg g^{-1} fw, by the methods of Nagata and Yamashita (1992) and iMLR, respectively (Table 3.6). Differences became evident when the methods of Wellburn, Nagata and Yamashita, and iMLR were compared (Figure 3.3). The content of bCAR appeared stable for use of the equation systems as well as iMLR (Table 3.6), while the calculated content

Table 3.5.: Effect of fruit ripening on the content of chlorophylls in fw extracted from fresh tomato pericarp and determined by different methods of spectral analysis (μgg^{-1})[a]

	CHLa		CHLb	
Stage	Wellburn (1994)	iMLR	Wellburn (1994)	iMLR
4	15.84±8.30	12.80±6.80	3.13±1.40	3.71±1.78
6	1.10±1.20	0.81±1.10	–	0.30±0.41
7	0.20±0.60	0.10±0.40	–	0.38±0.33
8	0.40±0.60	–	0.60±1.00	0.59±0.36
10	–	–	0.26±1.30	0.43±0.43

[a] Values are average ± SE for each ripening stage (n = 15).

Table 3.6.: Effect of fruit ripening on contents of carotenoids in fw within tomato pericarp determined by different methods of spectral analysis ($\mu g g^{-1}$)[a]

	bCAR			LYC		LUT
Stage	Wellburn (1994)[b]	Nagata and Yamashita (1992)	iMLR	Nagata and Yamashita (1992)	iMLR	iMLR
4	12.96±6.45	19.98±10.31	1.47±5.39	3.44±2.54	0.57±1.20	19.72±9.43
6	59.98±25.74	36.48±5.80	34.52±9.52	46.82±25.47	37.58±24.46	16.81±5.25
7	70.49±27.27	33.51±7.87	33.97±11.95	58.26±25.67	49.78±24.74	13.37±5.30
8	99.75±30.59	38.31±8.42	40.65±10.42	86.17±29.89	76.68±30.18	12.33±5.84
10	157.92±28.94	47.42±11.52	48.49±14.80	141.72±25.32	132.35±24.47	11.51±5.00

[a] Values are average ± SE for each ripening stage (n = 15).
[b] CAR_{total}.

Table 3.7.: Correlation of *in-vivo* measured tomato pigment contents determined by iMLR and wet chemical analyses

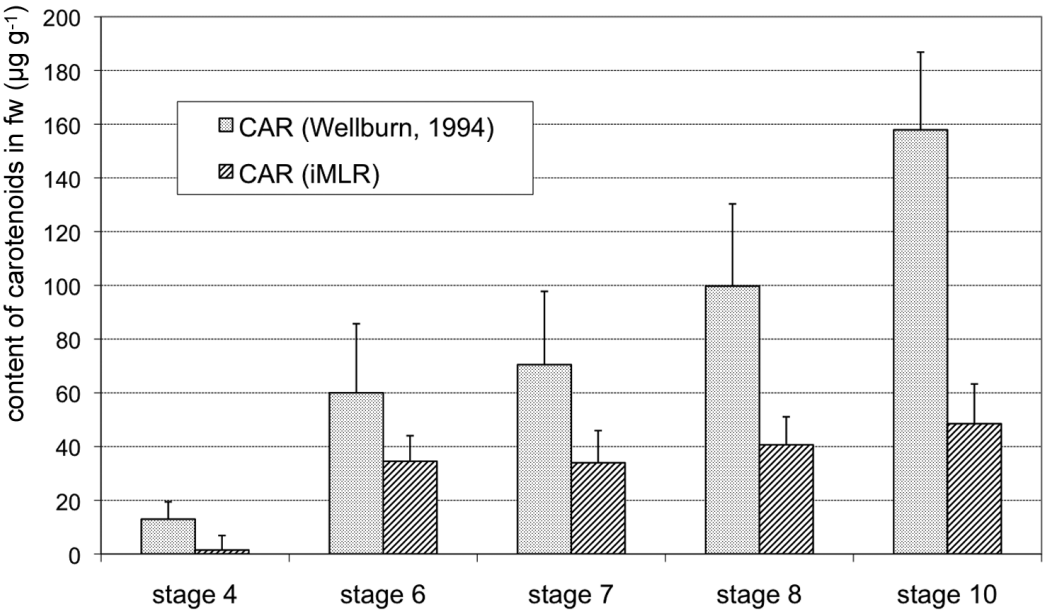
Test	LYC	bCAR	CHLa	CHLb	Total Chlorophylls
r^2	0.67	0.03	0.82	0.84	0.83
$rmse$ (mg L^{-1})	16.26	33.98	3.80	0.81	4.47
$rmse$ (%)	23.34	85.71	41.71	37.42	39.65
Bias	-3.62	-38.60	8.83	1.26	10.73

of bCAR ranged from 12.96 $\mu g\, g^{-1}$ fw to 157.92 $\mu g\, g^{-1}$ fw for use of Marr et al. (1995). The values show enhanced measuring uncertainty when equation systems with only one specific extinction coefficient for bCAR are applied. Concluding, more than two determinants are needed to calculate bCAR, LYC, and LUT (CAR_{total}) in tomato.

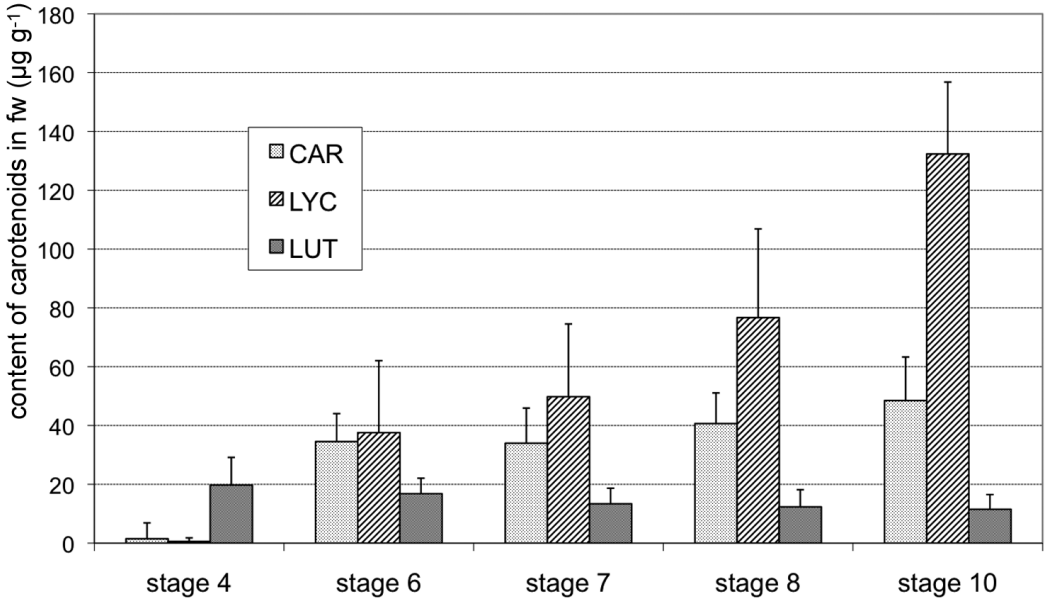
Furthermore, a small range of LUT content was observed at the measured ripeness stages, which was detectable solely with iMLR. Here, the perturbation of unexpected carotenoids composition on the calibration with common methods became evident. The content in fresh fruit sample was determined with 19.7 $\mu g\, g^{-1}$ at ripeness stage 4 and decreased during maturity stages to 11.5 $\mu g\, g^{-1}$. After comparison of the average values of tomato pigment contents at different maturity stages, it can be concluded that the highest LYC and bCAR contents were found, as expected, in tomatoes of highest maturity stage (stage 10). Moreover, the method developed allowed LUT to be detected in extracts.

C. *In-vivo* measured pigments

Sum spectra (400-800 nm) of tomato fruits at different ripeness stages are shown in Figure 3.4. All measurements were directly performed on fresh tomatoes recorded non-invasively in reflectance mode by using an integrating sphere. Absorption maxima appeared at 565 and 681 nm. The local maximum at 681 nm was attributed to the absorption of chlorophylls.

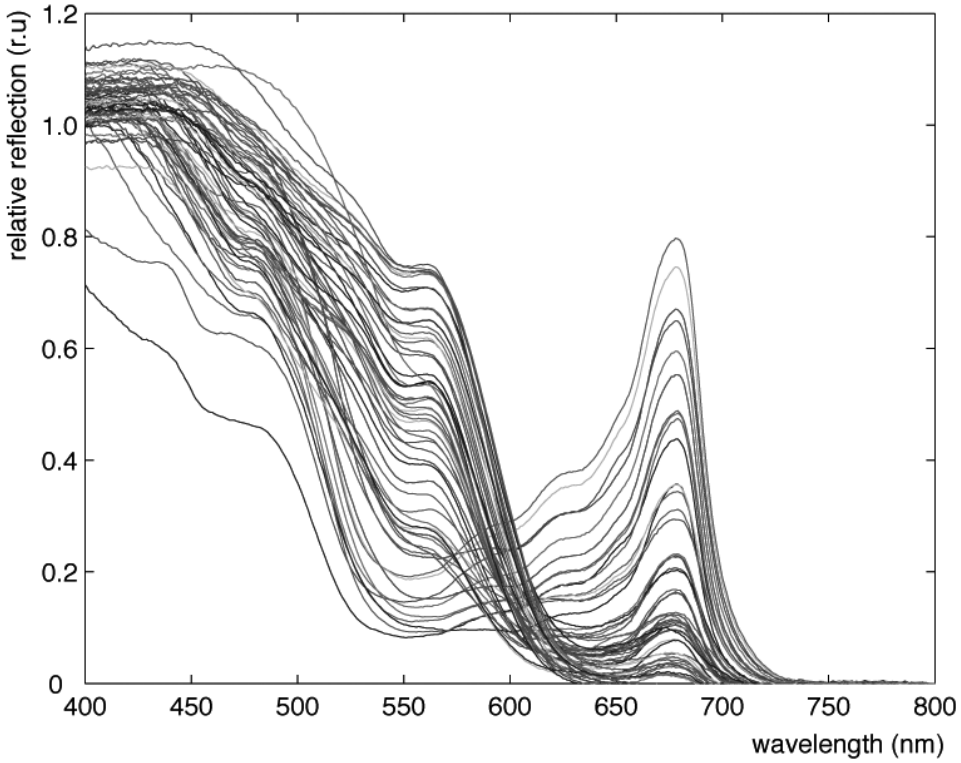


(a)

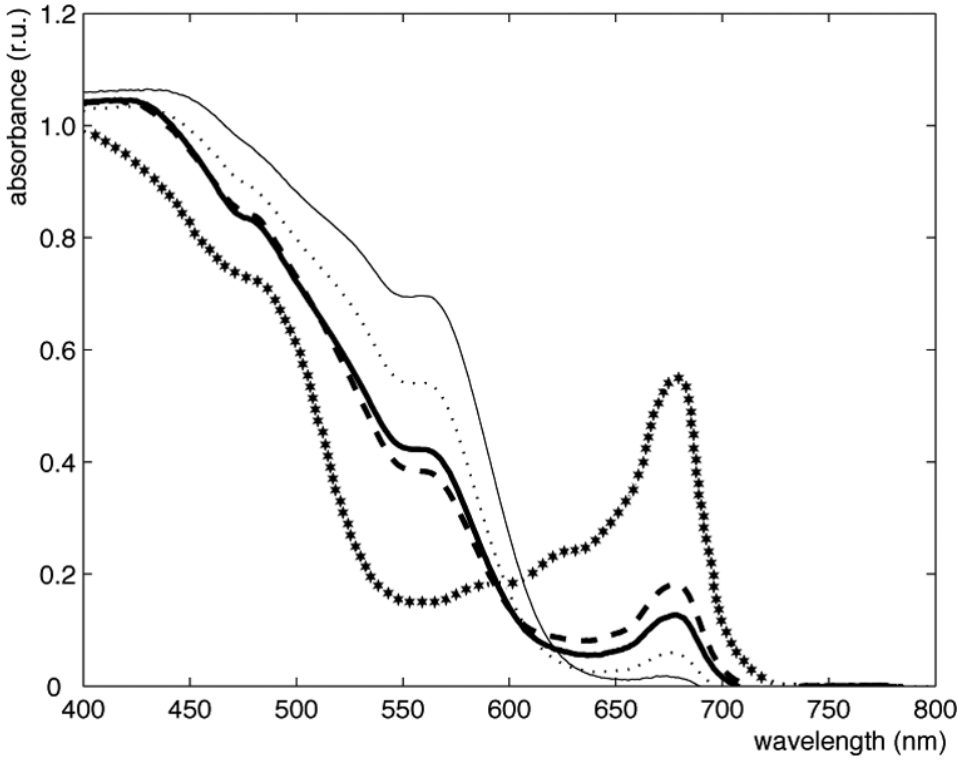


(b)

Figure 3.3.: Content of (a) bCAR and (b) LYC and LUT in the pericarp determined by iMLR and the method of Nagata et al. (2007).



(a)



(b)

Figure 3.4.: (a) Non-invasively measured VIS spectra (reflection mode by using integrating sphere) of tomato samples at different ripeness stages. (b) Averaged sum spectra of stage 4 (asterisks), stage 6 (dashed), stage 7 (solid), stage 8 (dotted), and stage 10 (thin solid).

With increased maturity stage, the absorption decreased. Since the local maximum at 565 nm can be separated at higher ripeness stages, it was attributed to the absorption of LYC. Using the method developed, CHLa and CHLb contents were separately analyzed in tomatoes of different maturity stages. The correlation of wet chemical results and calculated data based on spectral diffuse reflectance data from tomato tissue was high. The coefficient of determinations were $r^2 = 0.82$ and $r^2 = 0.84$ for CHLa and CHLb, respectively (Table 3.7).

3.1.7. Conclusion

An iterative algorithm (iMLR) was developed to separate overlapping absorption spectra of carotenoids and chlorophylls based on the known absorption coefficient profiles of pigment standards. The method was verified by dilution series at adjusted compositions of pigment standards. The method presented provides a feasible tool for a quantitative separation of single pigment absorption based on a spectrophotometrically measured sum spectrum. Thus, it makes a contribution for laboratory analyses and evaluation of *in-vivo* measured spectral data. Further substantial influences of scattering effects remained unconsidered and will be included in future studies.

3.2. Application

3.2.1. Corresponding paper

Chapter 3.2 is consistent with the following publication (paper II):

Pflanz, M., Mudau, N., and Zude, M. (2010). Separation of absorption coefficients from ripeness-related fruit pigments in stored mango. Erwerbs-Obstbau, 52(1):1–9.

Status: Received 11 February 2010; accepted 5 March 2010; published (online) 31 March 2010

3.2.2. Abstract

The maturity of horticultural produce is well correlated with the breakdown of green and synthesis of yellow skin pigments. In particular, the specific light absorption of intact tropical fruit is characterised by varying contents of chlorophylls, carotenes and xanthophylls. Accordingly, the fruit ripeness could be precisely and cost-efficiently determined by non-destructive spectral readings from UV/VIS wavelength ranges. In the present study postharvest observations on varying contents of chlorophylls, carotenoids and xanthophylls in ripening mango

fruit (*Mangifera indica* L. 'Kent') were used to evaluate a new method of analysing spectral data by an iterative MLR.

The main objective was to establish this method as a laboratory application for analysing fruit extracts in organic solvents containing carotenoids as wells as xanthophylls. It should be applied further in non-destructive quality tests on fruit and vegetables. In comparison with commonly used sets of linear equations calculating major compounds, the iterative MLR provides determinations of individual variable mango pigments. Varying contents of CHLa and CHLb as well as bCAR and VIO can be calculated in extracts of mango pigments by using iMLR. In samples from exocarp (skin) of overripe fruit the content of VIO was 18.04 $\mu g\, g^{-1}$ dw and thus was significantly higher compared to samples from unripe fruit exocarp (8.63 $\mu g\, g^{-1}$ dw). As the content of bCAR did not change significantly under adjusted postharvest storage conditions, its level was significantly higher in overripe fruit. The content of CHLa and CHLb was 27.53 $\mu g\, g^{-1}$ dw and 7.56 $\mu g\, g^{-1}$ dw respectively in unripe fruit. It significantly decreased during the ripeness of fruit and was 12.40 $\mu g\, g^{-1}$ dw and 4.04 $\mu g\, g^{-1}$ dw respectively in overripe fruit. Due to the high variation, a monitoring of individual pigments could help to improve the prediction of the quality of tropical fruits during their development on the plant and in the postharvest chain.

Within the limits of light scatter effects, the iMLR was also applied for the analysis of non-destructively recorded fruit spectra. But with respect to diffusive reflectance in biological tissue, considerable corrections to varying optical properties are needed. The measuring uncertainty was consequently only low for chlorophyll, but high for single carotenoids.

3.2.3. Introduction

Quality and shelf-life are vital characteristics of horticulture crops that depend on environmental conditions at growing and the physiological constitution of fruit at harvest. External attributes, such as size, shape and colour are in fact useful indicators to estimate the fruit's development, but are insufficient to cover all nutritional and physico-chemical criteria. Indeed, colour changes are closely-related to the physiological stage during the fruit's development, but less responsible for instruments with low optical sensitivity. According to this, the accurate quantification of individual chromophors (pigments) could help to obtain more reasonable indicators of fruit quality with relation to the optimal harvest time and postharvest treatments. In practice, product quality tests on apples and tomatoes have been applied in sorting lines, which is typically based on RGB imaging. Until now this method generates inadequate data, even though it is known that the colour of biological material is affected through light absorption by individual pigments. Thus, the content of major pigments has been more specifically determined through non-destructive spectroscopy, and was shown as well correlated with fruit ripeness (Zude, 2003; Baranska et al., 2006).

However, the analysis of data from spectral recordings on living plant tissue or liquid

pigment extracts is complicated due to light scattering and coinciding absorbance. Taking into account the latter, spectroscopic measurements always result in sum signals from all individual light absorbances of each pigment involved in a natural mixture. In unripe green fruit chlorophyll strongly absorbs light from short and long wavelength ranges, which leads to colour-masking effects. As a result, orange or red pigments of lower content, typically occurring in unripe fruits, become invisible for instruments with low sensitivity. To provide more specific information about fruit quality, shelf-life and optimal harvest time simple tissue extracts or even non-destructive measurements, need to be established as a more suitable sampling technique.

Mangos are important tropical crops with limited shelf-life but high nutritional value. Their secondary metabolites such as carotenoids have been widely studied (Ajila et al., 2007b) and pointed out to be of high relevance for the human diet in terms of high provitamin A activity (Pelz et al., 1998). Even though up to 16 (oxygenated) carotenoid derivatives (xanthophylls) were identified in fresh mango fruit, bCAR was the most frequently occurring carotene with 60 % of CAR_{total} content (Jungalwala and Cama, 1963; Cano and Deancos, 1994; Mercadante and Rodriguez-Amaya, 1998; Ornelas-Paz et al., 2007). Nevertheless, it was shown through high-grade carotenoid analysis that numerous other carotenoids vary during the ripening of mango fruit (John et al., 1970; Godoy and Rodriguez-Amaya, 1987; Wilberg and Rodriguezamaya, 1995).

However, because such laboratory analyses, like high performance liquid chromatography (HPLC), are expensive and time-consuming, simpler methods of liquid chromatography have been established. Linear equations are typically used there to analyse spectral data from liquid mango fruit extracts. In this regard, the content of CAR_{total} was shown to be well correlated with organically solved red and yellow pigments (John et al., 1970; Ajila et al., 2007b; Ribeiro et al., 2007), but poorly correlated with individual carotenes and xanthophylls.

Instead of applying fixed instruments, non-destructive (portable) techniques are available and have been used to determine contents of major pigments on plants. Nevertheless, an accurate determination of individual pigments (i.e. carotenoids) is still required. Besides complex light-scattering effects in biological tissues, the use of the Lambert-Beer law for the quantification of pigment contents after chemical extraction is limited, due to a strong spectral overlap of light absorption by different plant pigments being present in a mixture. Chlorophylls as well as carotenoids generate strongly coinciding signals within the UV wavelength range (Comar and Zscheile, 1942; Wellburn, 1994; Nagata, 2007). Thus, these sum signals need to be separated using spectral signatures of each carotenoid to minimise the error of estimation.

In the present study, a new approach was applied to determine chlorophylls and carotenoids in fresh and postharvest ripened mango fruit. Based on an iterative MLR algorithm, spectral signatures of CHLa and CHLb, bCAR, LUT and VIO were used to analyse spectral sum signals from chemically extracted mango pigments. This method was validated by Pflanz and Zude (2008) . In comparison with conventional methods, which show high accuracy

only for chlorophylls and CAR_{total} content, CHLa and CHLb as well as bCAR, LYC and LUT were more specifically determined by iMLR with high reliability and low $rmse$. The iterative MLR was further applied to analyse non-destructive spectral readings of ripening mango samples. With regard to other post-processing data analysis, non-destructive spectroscopy has successfully been applied to determine the ripeness-related content of pigments in apples, tomatoes and carrots (Zude, 2003; Baranska et al., 2006; Zude et al., 2008b).

3.2.4. Material and methods

The study was carried out on mango fruit of cultivar 'Kent'. In terms of ripeness-related changes of external and internal fruit characteristics, varying contents of CHLa, CHLb, bCAR and VIO, as well as soluble solids and organic acids were measured. A total of n=30 fruit were harvested in an experimental orchard at the University of Limpopo (Turfloop Campus, South Africa) and transported to the food science laboratory of Leibniz Institute for Agricultural Engineering Potsdam-Bornim (ATB, Potsdam, Germany) within 48 hours. Depending on their skin colour, some overripe or decayed fruit were rejected. Following this, 10 fruits were ripened to table-ripeness for 5 days under adjusted storage conditions at 20° C air temperature and 85 % relative humidity. Another 10 fruits were harvested 30 days later and classified as overripe samples. All samples were monitored initially and following once a day by non-destructive readings. Diffuse reflectance was measured in UV/VIS wavelength ranges (350-1400 nm) using an integrating sphere (Lambda 950, Perkin-Elmer, USA). Destructive measurements were carried out at each variant immediately or after removal from storage.

Analysis of pigments. Optical measurements, which were non-destructively performed on the whole fruit, were compared to chemically determined contents of chlorophylls and carotenoids. For chemical analyses, samples of 2 and 10 mm thickness were taken from the exocarp (peel) and mesocarp (pulp) respectively using a cork borer of 18 mm diameter. Next, the tissue samples were homogenised (UltraTurax T18, IKA Works, USA) by continuously adding 80 % acetone (Roth, Germany) and a small amount of calcium carbonate. Such organically dissolved pigments were separated from the remaining tissue using a G3 filter and filled up to 100 mL of acetone-pigment mixture. Chromophoric compounds were transferred from the polar phase in acetone to the non-polar phase in diethyl ether by adding a small amount of water and 25 mL diethyl ether (Merck, Darmstadt). The concentration of chlorophylls and carotenoids was finally determined by spectrometric readings (Lambda 950, Perkin-Elmer, USA) in the VIS wavelength range from 350-850 nm and calculated according to Lambert-Beer's law and iMLR.

Sugars. Tissue samples were mechanically squeezed and the juice was frozen and stored at -18° C. To isolate mono- and disaccharides, after protein degradation via Carrez-reagent, HPLC (Dionex, USA) was performed using a Eurokat H column (300 mm x 8 mm, 10m) and a mobile phase 0.01 N H_2SO_4 at a rate of 0.8 mL min^{-1} at 63 Pa. A refractive index detector (RI-71, Shodex, Techlab, Germany) was calibrated with standards of glucose, fructose, and

sucrose (Merck, Germany).

SSC and acids. The SSC was measured in values of °Brix, using a digital refractometer (PR-1, Atago Co., Japan). TA was determined by titrating the juice with 0.1 N NaOH, and the data were expressed as a citric acid equivalent.

Data evaluation. Variations of individual pigment content were determined using a self-developed MLR analysis (iMLR Version 1.55, ATB, Germany), which fits spectral pigment signatures (profiles) as a function of recorded sum signals by minimising the mean squared error (Pflanz and Zude, 2008). A stand alone application of iMLR was developed, which provides a graphical user interface. It enables basic operations to prepare raw data for multiple regression analysis including the import of raw spectra and reference data, wavelength range limitation as well as the export of results. A baseline correction is automatically performed at the given wavelength. The algorithm is available for scientific purposes at http://www.atb-potsdam.de.

In default mode, iMLR provides suitable pigment profiles of CHLa and CHLb, bCAR, LYC and LUT to analyse tomato, melon or papaya extracts. Extended functionality is given by loading optional spectral profiles, such as used for analysing VIO or NEO in mango fruit (Figure 3.5). The application presented here is available for the analysis of liquid chromophoric solvents.

A comparative analysis of non-destructively recorded mango spectra was performed with iMLR and PLS analysis, using MATLAB (Version 7.4, The Mathworks Inc., USA) and the Eigenvector PLS-Toolbox (Version 3.4, Eigenvector Research, Inc., USA). To compare these results with those calculated by iMLR, a multivariate calibration of non-destructive readings and chemically determined contents of ripeness-related pigment change was conducted. Raw spectra were pre-processed by applying scaling and derivative methods eliminating noise and temperature-dependent wavelength shifts. A standard normal variate (SNV) transformation was additionally applied to remove multiplicative interferences of scatter and particle size (Dhanoa et al., 1994; Candolfi et al., 1999).

Statistical analysis was carried out using SPSS (Version 15, SPSS Inc., USA) to determine the variance of mean values. The homogeneity of variances was tested using Levene's test and significant mean differences were determined by T-tests at a significance level of $p < 0.05$.

3.2.5. Results and discussion

Destructive measurements

Sugar and acids. The presence of different sugars plays an important role in the mango fruit quality evaluations of consumers (Gonzalez-Aguilar et al., 2008). In agreement with

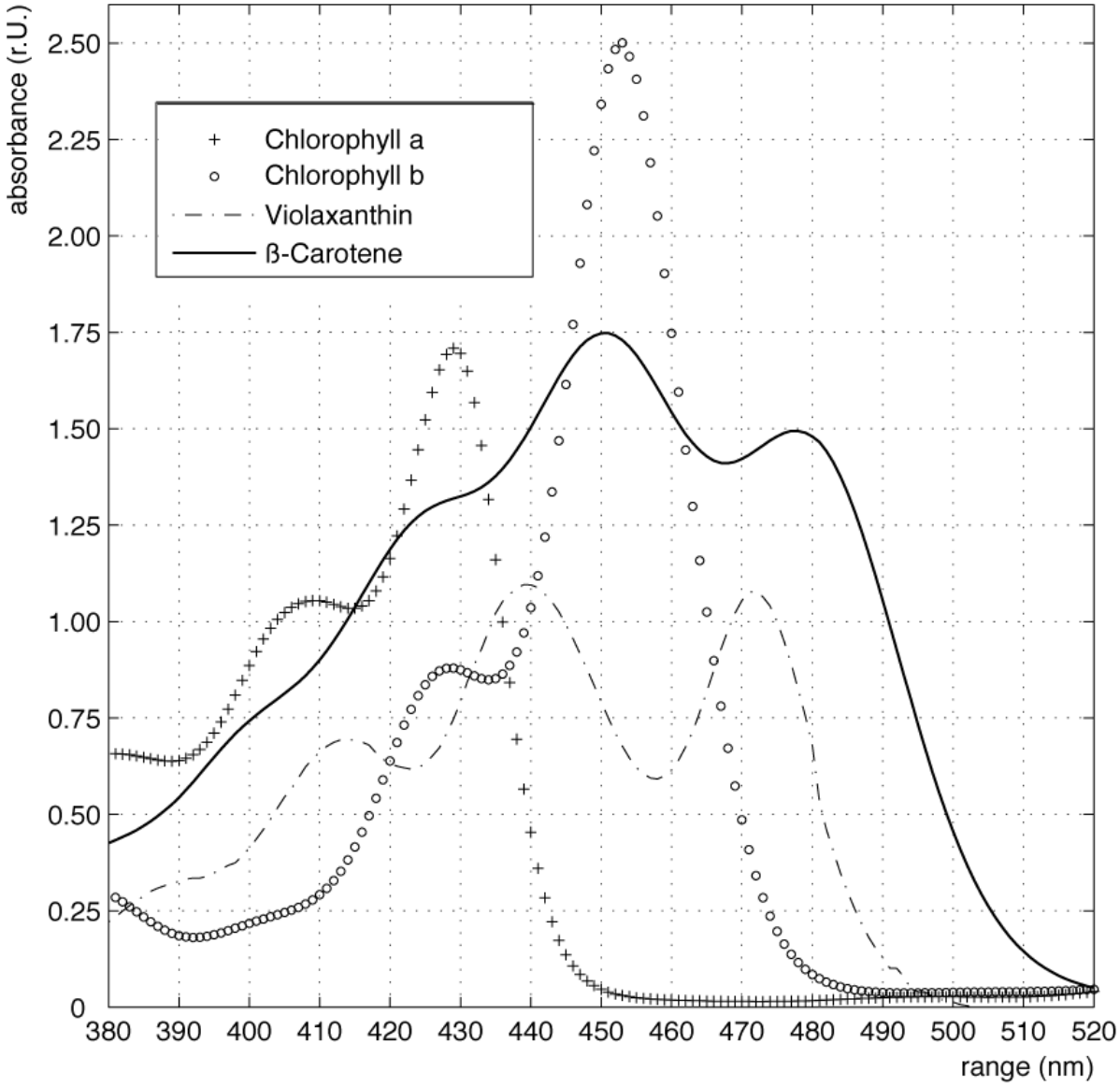


Figure 3.5.: Spectral profiles of bCAR (solid), VIO (dotted), CHLa (plus) and CHLb (circles) within the wavelength range of 380-520 nm.

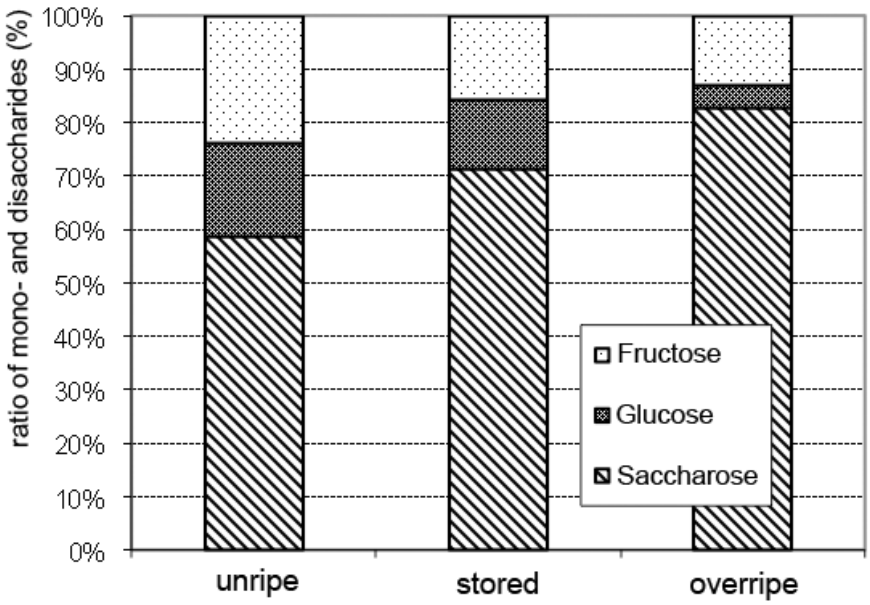
previous studies, mango fruits have a high variability in fruit sugar content depending on the cultivar as well as on their ripeness stage and storage conditions (Castrillo et al., 1992; Talcott et al., 2005; Léchaudel and Joas, 2006). Within the group of the three tested fruit sugars, the ratio of sucrose was the highest in all stages of ripeness (Figure 3.6a). In unripe fruit a mean value of 58.7 % was determined. The percentage of sucrose increased up to 71.2 % in stored fruit and was 82.7 % in overripe fruit. Fructose was catabolised during the period of fruit maturation. A decreasing content of 24 % was found in unripe fruit, 15.7 % in stored and 13.1 % in overripe fruit respectively. The lowest proportion compared to the total sugar content was found for glucose, which also decreased during fruit ripening.

In addition to the amount of sugars, the presence of fruit acids has a high impact on the consumer's senses. Therefore, the relation between sugars and acids (sugar-acid ratio) is essential for the assessment of fruit quality. Here, a varying ratio of sugars to acids (11.5 and 41.5) was found within the three ripeness stages analysed. In agreement with the results of Vasquez-Caicedo et al. (2005), fruits at medium ripeness stages were suitable for consumption and showed a sugar-acid ratio of 25.9 (Figure 3.6b). All mean values significantly vary at a level of 5 % ($p < 0.05$).

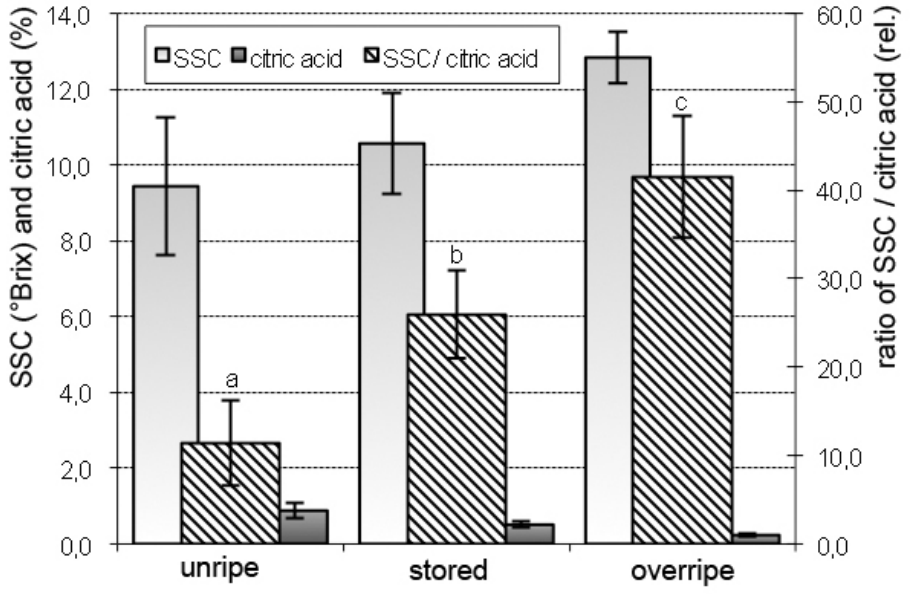
Pigment separation using iMLR. The analysis of destructively recorded spectra was carried out using equal equation systems according to Wellburn (1994) and the found data were compared to the results of iMLR. Previous studies to determine individual carotenoids, using standard solutions of pigments, have shown high coefficients of determination with less errors of measurement (Pflanz and Zude, 2008). In the solvents of pigment extracts from mango fruit, strongly coinciding effects were observed within the wavelength range of carotenoid light absorption between 350-550 nm (Figure 3.7). Using iMLR, spectral signatures of typical mango pigments could still be separated from the measured sum signals.

Pigments in exocarp. While no differences in the maturity stages of the samples were determined by the fruit's colour appearance, the variable amount of ripeness-related fruit pigments chemically separated from the exocarp of fruit was high. Table 3.8 shows the standard deviation of the calculations. The initial content of CHLa was 27.53 μgg^{-1} dw, with a statistical variation of 6.53 μgg^{-1} dw. It did not significantly vary until the end of the storage period. The initial content of CHLb in unripe fruit was 7.56 μgg^{-1} dw with no significant differences in postharvest ripened fruit at adjusted conditions (Table 3.8). Consequently, in terms of its chlorophyll content, unripe fruit cannot be differentiated from fruit stored for five days. In contrast, overripe fruit showed a content of 12.40 μgg^{-1} dw of CHLa and 4.04 μgg^{-1} dw of CHLb in peel respectively. Thus chlorophyll contents in overripe fruit differ significantly from unripe fruit.

Analyses which were carried out using iMLR showed a ripeness-related increase of bCAR and VIO content in mango peel (Table 3.8). The content of VIO was 8.63 and 9.41 μgg^{-1} dw in unripe and overripe fruit respectively, and thus significantly increased. The content of bCAR did not change significantly between unripe and postharvest ripened fruit. The amount of bCAR in the peel of unripe fruit was 6.65 μgg^{-1} dw and did not significantly



(a)



(b)

Figure 3.6.: (a) Variation of mono- and disaccharides and (b) varying sugar-acid ratios in ripening mango fruit. Significant mean differences are indicated by different letters ($p<0.05$).

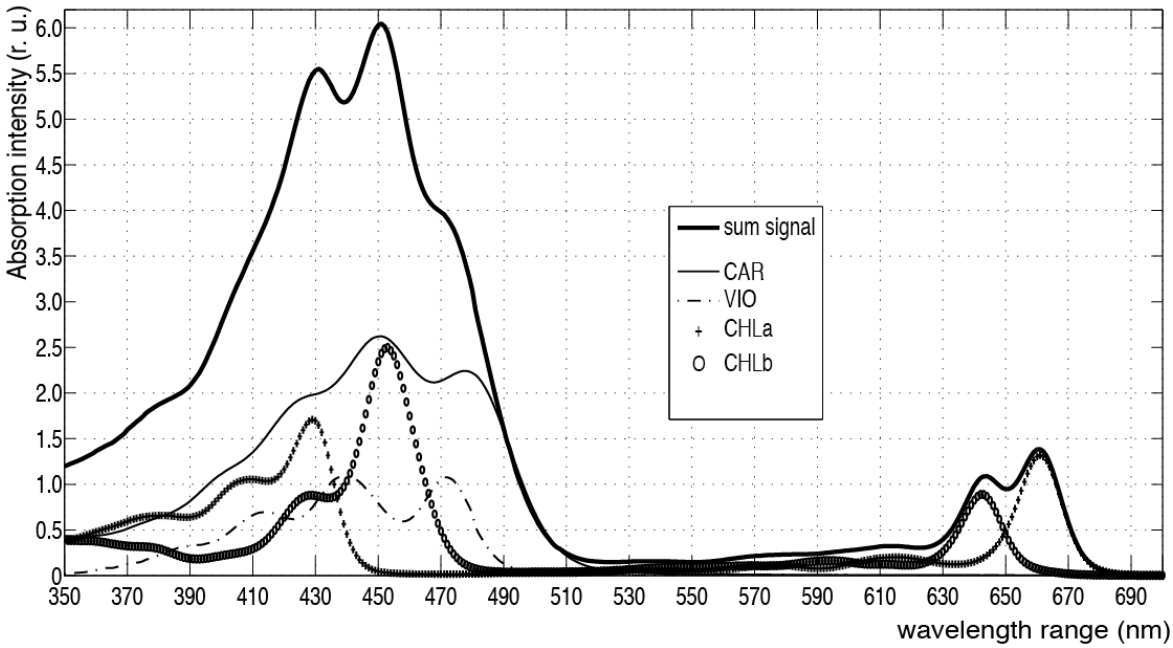


Figure 3.7.: Typical sum signal (thick solid) of spectral readings on liquid extracts of pigments, occurring in ripening mango fruit. At the stage of medium fruit ripeness shown here CHLa (plus) and CHLb (open circle) are still present in the mixture, while CAR (thin solid) and VIO (dotted) are significant increasing. A spectral separation of individual chlorophylls and carotenoids in the VIS wavelength range is provided by iMLR.

increase during storage. In contrast, a significant increase of bCAR (16.50 μgg^{-1} dw) was observed in overripe fruit (Table 3.8).

However, it was shown that even slight changes of individual pigments after short storage periods are detectable by means of iMLR. Due to minor benefits in terms of human diet, the changes of individual mango skin pigments are less studied. Regarding the ripeness stage of the fruit, varying contents of peel pigments in different mango cultivars have been observed by Ajila et al. (2007). As well as in the present study, CAR$_{total}$ contents were determined by the Wellburn method (1994). Consequently, no individual carotenoid contents were determined by Ajila et al. (2007). Nevertheless, it was found that the peel from fully ripe mango fruit of cultivar 'Raspuri' has a 4-8 times higher content of CAR$_{total}$ compared to unripe fruit (Ajila et al., 2007a). This has not been verified by the present study.

Pigments in mesocarp. In addition to the amount of pigments located in the exocarp tissue, varying contents of chlorophylls and carotenoids from the mesocarp were also analysed. It was shown that chlorophylls were no longer detectable at all ripeness stages. VIO showed the highest increase during the entire maturity period. The final measured mean content was 24.71 μgg^{-1} dw and thus comparable to other studies (Godoy and Rodriguez-Amaya, 1987; Mercadante and Rodriguez-Amaya, 1998). In unripe fruit, the content of VIO was 4.94 and 5.28 μgg^{-1} dw in stored fruit respectively. In contrast, the mean content of bCAR ranged from 2.07 μgg^{-1} dw in unripe, to 4.84 μgg^{-1} dw in postharvest ripened fruit and increased

Table 3.8.: Content of ripeness-related mango fruit pigments in μgg^{-1} dw chemically extracted and analysed using iMLR and linear equations[4]

	Exocarp			Mesocarp		
Pigments	unripe	stored	overripe	unripe	stored	overripe
CHLa[1]	27.53±6.53a	30.97±16.23a	12.40±4.25b	–	–	–
CHLb[1]	7.56±2.42a	8.91±3.53a	4.04±1.04b	–	–	–
CHLa[2]	30.65±8.75a	34.84±13.25a	11.95±7.60b	–	–	–
CHLb[2]	10.18±3.59a	11.53±5.07a	5.34±3.65b	–	–	–
bCAR[1]	6.65±1.73a	10.36±2.29a	16.50±4.75b	2.07±0.74A	4.84±1.91B	7.64±1.29C
VIO[1]	8.63±4.23a	12.61±3.56b	18.04±5.44c	4.94±2.94A	5.28±2.39A	24.71±4.44B
CAR^{3}_{total}	15.47±5.29a	29.10±7.46a	31.06±8.88b	7.01±3.63A	9.42±3.46A	32.34±5.71B
CAR^{2}_{total}	12.99±5.86a	17.22±3.51a	27.80±5.77b	7.90±3.03A	14.27±6.60A	64.95±15.63B

[1] Calculated by iMLR (Pflanz and Zude, 2008); [2] Calculated by the Wellburn method (1994); [3] Cumulative
[4] Significant mean differences are indicated by different letters ($p<0.05$)

to 7.64 μgg^{-1} dw in overripe fruit. Through the use of iMLR, it was observed that the mean differences of bCAR content change only slightly but significantly between stages of unripe and stored fruit. In contrast, no significant variation was found for VIO at this early maturity stage (Table 3.8). Also, no significant differences in CAR_{total} contents were found at the immature and the medium stage of ripeness. Finally, no chlorophylls were found in mesocarp samples.

The analysis being carried out was improved by the concept of iMLR, which uses full spectral profiles to accurately separate individual pigment absorption spectra from sum signals. The principle of spectral separation is illustrated in Figure 3.7. The results of the present study have been confirmed through previous studies on freshly cut mango samples of different cultivars which were stored at comparable postharvest conditions (Gonzalez-Aguilar et al., 2008). It was shown that the content of bCAR significantly increases during the first three days of storage at 5° C air temperature. In addition, a significant change of bCAR content was observed at room temperature. Xanthophylls were not considered in this study. In contrast, Gonzales-Aguilar (2008) suggested that the content of bCAR remains constant in mango fruit after 12 days of storage at 5° C.

Non-destructive measurements

Size. The average fw of fruit was 380 g. The average mango fruit size was 115 mm at longitudinal distance and 85 mm at equatorial diameter.

Spectroscopy. The analysis of non-destructive spectral readings was performed after raw-spectra pre-processing, using a multivariate regression analysis as well as iMLR. A PLS regression was used to build calibration models, on the basis of diffuse reflection spectra and chemically determined pigment contents of CHLa, bCAR and VIO. All PLS calibration performance results are summarised in Table 3.9. In comparison with different pre-processing methods, improved calibration models were found for derivative conversion and SNV.

Table 3.9.: Summary of PLS regression statistics for non-destructive UV/VIS models of varying main pigments in intact mango fruit according to the number of latent variables (LV) and in comparison to different pre-processing methods (raw = no pre-processing, derivative spectra, SNV = Standard Normal Variate) at relevant wavelength ranges.

	Pre-processing:	raw spectra (%R)			derivative spectra f' (%R)			SNV		
		r^2	LV	$\%\,rmsec$	r^2	LV	$\%\,rmsec$	r^2	LV	$\%\,rmsec$
Light absorption at relevant wavelength	CHLa	0.60	2	33.35	0.69	2	29.53	0.59	2	33.71
	600-750 nm	0.69	3	29.29	0.71	3	28.28	0.68	3	29.87
		0.70	5	28.69	0.85	5	20.24	0.73	5	27.37
		0.85	9	20.54	0.95	9	12.02	0.87	9	18.70
	bCAR	0.46	2	24.45	0.42	2	25.29	0.34	2	26.97
	350-600 nm	0.47	3	24.14	0.54	3	22.53	0.35	3	26.74
		0.56	5	21.98	0.69	5	18.57	0.51	5	23.30
		0.63	9	20.18	0.95	9	7.78	0.60	9	21.01
	VIO	–	–	–	0.38	2	39.09	0.28	2	42.09
	350-600 nm	0.56	3	33.03	0.56	3	32.89	0.30	3	41.59
		0.38	5	39.01	0.80	5	22.02	0.44	5	37.03
		0.56	9	33.03	0.97	9	8.95	0.58	9	32.26

The derivative pre-processing of diffuse reflection required less LV and showed higher coefficients of determination for the prediction of chlorophyll a. The performed wavelength truncation led to a higher accuracy and a lower root mean square error of calibration ($rmsec$). This truncation was limited for CHLa (600-750 nm) and CAR_{total} (350-600 nm). A similar approach was used by Delwiche (2008). Finally, the error of prediction was 0.48 (LV=9) and 1.17 (LV=2). Compared to the calibration after SNV pre-processing, no significant lower errors were observed for the prediction of chlorophyll a.

The analysis of non-destructive data required no additional calibration using the iterative MLR. However, the decomposing of CHLa absorption from spectral sum signals was inaccurately pointed out in a low coefficient of determination (r^2=0.23). The coefficient of determination could be improved (r^2=0.64) if wavelength shifts at the chlorophyll absorption range were also estimated by the iterative algorithm (Table 3.10). Nevertheless, the relative error was still high ($\%rmse$ = 17.92). Spectral variations caused by bCAR or VIO were not detectable in *in-situ* recorded data.

3.2.6. Conclusion

Spectrometrically measured sum signals from organic extracts from mango exocarp and mesocarp were analysed with respect to varying contents of CHLa and CHLb (not found in mesocarp), bCAR and VIO. The content of CHLa and CHLb determined by conventional methods

Table 3.10.: Coefficients and errors of the correlation between non-destructively and destructively calculated contents of chlorophyll in ripening mango fruit. In addition, the ripeness-related wavelength shift of chlorophyll was determined by iMLR and used as corrective factor.

iMLR	Content of chlorophyll (destructive)		
(non-destructive)	r^2	$rmse$	$\%\,rmse$
Chlorophyll + shift	0.64	0.91	17.92
Chlorophyll	0.23	0.07	1.38
shift	0.60	2.21	43.52

and iMLR showed no significant differences. CAR_{total} ranged in amounts, comparable to those found in previous studies. Through the use of iMLR, it was shown that an increase of CAR_{total} in mango exocarp is essentially caused by a significant increase of VIO, while the content of bCAR did not significantly differ between unripe and postharvest ripened fruit, stored at 20°C and low relative humidity levels. Due to the inhomogeneity in raw material, this assumption needs to be critically challenged and validated.

The iterative working algorithm provided a quantitative separation of pigments, typically found in ripening mango fruit, even if their spectral light absorption strongly coincided. Compared to commonly used equation systems for separating coincided spectral signals, the implementation of iMLR could improve pigment analyses of horticultural products. The iMLR may allow an analysis of individual pigments in other fruit species as well. For destructive measurements which could also focus on the identification of unknown quality-determining compounds, high-precision analytical methods like HPLC are more suitable.

To analyse non-destructive data, no calibration was required using iMLR. Spectral variation caused by varying pigment contents was determined based on spectral profiles of pigments expected in senescent fruit tissue. In addition, coinciding spectral effects, scatter and ripeness-related wavelength shifts were observed. These substantial influences are still unconsidered and will be included in following studies. Consequently, future experiments should prove the adaptability of this method to evaluate *in-situ* recorded spectral data.

3.2.7. Acknowledgement

We would like to acknowledge all scientific staff and co-workers of the Department of Horticultural Engineering, ATB for their help in realising the experiments and Nixwell Mudau from the Department of Plant Production, University of Limpopo for donating the fruit.

3.3. Scatter correction

3.3.1. Corresponding paper

Chapter 3.3 is consistent with the following publication (paper III):

Zude, M., Pflanz, M., Spinelli, L., Dosche, C., and Torricelli, A. (2011). Non-destructive analysis of anthocyanins in cherries by means of lambert-beer and multivariate regression based on spectroscopy and scatter correction using time-resolved analysis. Journal of Food Engineering, 103(1):68–75.

Status: Received 19 December 2009; Received in revised form 31 August 2010; Accepted 30 September 2010; Available online 28 October 2010

3.3.2. Abstract

In high-value sweet cherry, the red coloration - determined by the anthocyanins content - is correlated with the fruit ripeness stage and market value. Non-destructive spectroscopy has been introduced in practice and may be utilized as a tool to assess the fruit pigments in the supply chain processes. From the fruit spectrum in the VIS wavelength range, the pigment contents are analyzed separately at their specific absorbance wavelengths.

A drawback of the method is the need for re-calibration due to varying optical properties of the fruit tissue. In order to correct for the scattering differences, most often the spectral intensity in the VIS spectrum is normalized by wavelengths in the NIR range, or pre-processing methods are applied in multivariate calibrations.

In the present study, the influence of the fruit scattering properties on the VIS/NIR fruit spectrum were corrected by the effective pathlength in the fruit tissue obtained from time-resolved readings of the distribution of time-of-flight (DTOF). Pigment analysis was carried out according to the Lambert–Beer law, considering fruit spectral intensities, effective pathlength, and refractive index. Results were compared to commonly applied linear colour and multivariate PLS regression analysis. The approaches were validated on fruits at different ripeness stages, providing variation in the scattering coefficient and refractive index exceeding the calibration sample set.

In the validation, the measuring uncertainty of non-destructively analyzing fruits with VIS/NIR spectra by means of PLS or Lambert–Beer in comparison with combined application of VIS/NIR spectroscopy and DTOF measurements showed a dramatic bias reduction as well as enhanced coefficients of determination when using both, the spectral intensities and apparent information on the scattering influence by means of DTOF readings. Corrections

for the refractive index did not render improved results.

3.3.3. Introduction

Assuring healthy human nutrition and improving the economic success of farmers producing fresh fruit and vegetables are priority targets in the context of current global changes. There is general consensus that especially in agriculture new innovative technologies are needed for appropriate process management. In presently developing technologies, optical methods in particular are principally suitable for onsite analysis. Spectroscopy in NIR as well as VIS wavelength ranges can serve as a feasible tool for non-invasive fruit analysis due to available miniaturised light sources as well as accurate, robust, and inexpensive spectrophotometer modules. The compounds have been implemented in high-end colorimeters, handheld units for spectroscopic analyses, and sorting lines (Ozaki et al., 2006; Garcia and Blasco, 2009). Based on the physics of NIR spectroscopy, a detailed review for agricultural and food products was presented by Birth and Hecht (1987) and, more recently, focusing on the initial data processing in fruit analyses, by Nicolai et al. (2007).

The VIS part of the spectrum provides information on the pigment contents. Spectra obtained non-destructively from the fruit sample are frequently analysed in terms of colour using various colour spaces. Since this paper uses sweet cherry as a model fruit, the literature was searched for correlations of spectral readings respective to fruit quality and ripeness stage. Colour readings for grading fruits are evaluated regarding the external appearance recognized by the consumer. High correlation of colour values in *L*a*b** colour space and the anthocyanin contents of cherries were presented in recent papers (Serrano et al., 2005; Goncalves et al., 2007; Manganaris et al., 2007; Serrano et al., 2009). It was shown that variations of fruit pigment composition in different cultivars and colours correlate with the anthocyanins content and anti-oxidative capacity, with higher values in cultivars with visually dark appearance (Karlidag et al., 2009). Ripening-related enhanced colour appearance and corresponding anthocyanin content resulted in decreased values of *L**, *a**, and *b** during fruit development, shortly before harvest time at the tree (Kovacs et al., 2009) as well as in postharvest (Serrano et al., 2009).

However, it might be assumed that the apparent colour data can be influenced by other fruit pigments than anthocyanins such as chlorophylls and carotenoids, showing absorption at coinciding wavelengths. As an alternative, specific indices or whole spectra analyses have been introduced in practice and may serve as a more reliable method for measuring the cultivar- and maturity-related pigment development. From the fruit spectrum in the VIS wavelength range, the pigment contents are analysed separately at their specific absorbance wavelengths (Knee, 1972; Nagata and Yamashita, 1992; Pflanz and Zude, 2008).

Using VIS spectral readings of intact horticultural produce, the specific absorption characteristics can provide sensitive information on the changes in pigment contents. The data

point to physiological stages such as the chlorophyll-related ripeness in apple (Merzlyak et al., 1999; Zude, 2003; Herold et al., 2005; Peirs et al., 2005; Solovchenko et al., 2005), and can be used to assess the nutritional value of the product such as the content of native carotenoids in carrots (Zude et al., 2007). In this field of pigment detection, research in canopy remote sensing rendered the initial impulses (Penuelas et al., 1995; Gamon et al., 1997; Richardson et al., 2002). Several indices have been established in feasible applications, mainly for chlorophyll analysis, but also anthocyanin readings.

A drawback in the analysis of fresh, rapidly developing produce appears due to changes in the chemical composition as well as texture of the fruits. Researchers as well as developers of commercial VIS/NIR spectrophotometry equipment face difficulties with the robustness of calibration based on apparent spectral intensity, $I_R(\lambda)$. In fruit samples, we have to deal with the absorption coefficient at the wavelength under question, $\mu_a(\lambda)$ that provides quantitative information on the molecule content under question, as well as multiple scattering during the photon transport in the tissue. It can be assumed that depending on the phenotype of the fruit the scattering coefficient, μ_s, as well as the directional anisotropy factor, g, are varying. Results from simulations show that in some cases g has a major effect on the optical properties of kiwi fruit at different ripeness stages (Baranyai and Zude, 2009), while in apples the differences in g during fruit development appear marginal (unpublished data). The variation in μ_s is in any case a major factor, influencing the result when applying a calibration model. The resulting need for re-calibrations heavily limits the introduction of the sensors in practice.

The differences in the scattering properties actually have an impact on the effective pathlength, *L*s*, that the photon is traveling in the tissue on its way from the light source to the detector. If the scattering coefficient and, consequently, the effective pathlengths would be known, the Lambert–Beer law might be applied for pigment, c_i, analyses in intact fruit, such as presently carried out in the laboratory on fruit extracts:

$$log[100/I_R(\lambda)] = \sum c_i \epsilon_i L^*s. \tag{3.14}$$

It is accepted to estimate the effective pathlength of photons traveling through a sample by means of the reduced scattering coefficient, $\mu_s' = 1/L^*s$ (Cubeddu et al., 1999). While it appears difficult to obtain information on μ_s and g separately, the reduced scattering coefficient, $\mu_s' = (1-g)\mu_s$, can be measured by means of DTOF readings.

The time-resolved DTOF readings consist of injecting a short (picosecond) light pulse into the fruit to be characterized, while the transmitted or reflected photons are detected in a certain distance from the injector after traveling in the pericarp of the fruit. Attenuation and delay in pulse shape are due to the absorption and scattering events occurring into the sample, and an estimate of the optical parameters can be derived, based on a suitable model of light propagation, i.e. diffusion theory or Random Walk model (Patterson et al., 1989;

Contini et al., 1997; Cubeddu et al., 1999). Applying DTOF the μ_a can be uncoupled from the μ_s' (Jacques, 1989; Patterson et al., 1989; Arridge et al., 1992). A pre-assumption for application of DTOF in fruits is that the $\mu_s \gg \mu_a$ and the sample is large enough to avoid photon loss at the boundary of the sample. This is certainly not the case in fruits. However, predictions of the fruit SSC (Tsuchikawa and Hamada, 2004) and maturity-related chlorophyll content (Zerbini et al., 2006) were approached already by means of DTOF analyses.

Coming back to our problem of varying pathlength in the fruit samples and resulting perturbation in non-destructive analysis. It appears to be reasonable – even with expected high uncertainty of DTOF in cherries – to propose that the scattering influence on the apparent intensities in the fruit spectra can be corrected: knowing the spectral intensities at the specific absorption passband of the pigments as well as the effective pathlength by means of empirical DTOF data. Subsequently, a robust calibration on a certain pigment should be possible.

As an additional uncertainty, the refractive index of the sample has an impact on the apparent data from DTOF analysis due to varying boundary conditions (Contini et al., 1997). During fruit development the refractive index is changing and the influence of the variation in the refractive index on the calculation of the effective pathlength was not clear. Therefore, the non-destructive reading of the refractive index on the fruit material was approached as an additional step in sensor fusion in the present study. The total internal reflectance fluorescence (TIRF) microscopy was selected for this purpose, which is an emerging and effective technique for examining properties in the living cell structure (Oheim and Schapper, 2005). The method was chosen due to the opportunity for non-destructive readings. The study was aimed at evaluating an improvement in pigment analysis by means of the application of the actual refractive index.

The approach was compared with the established corrections by means of indices or multivariate data pre-processing. Indices use specific absorption passbands of the molecule corrected by NIR passband in the so-called "optical window". In the optical window almost no plant compound absorbs photons, and therefore, all variation appears due to scattering differences. Alternatively, adapted data pre-processing in whole-spectra analyses can be applied such as MSC, SNV, or working on derivatives. More recently orthogonal signal correction and further developed method as direct orthogonal signal correction were proposed for removing information not relevant to the absorption correlated with the molecule content under question. While having the advantage of being easy to apply methods of dealing with the problem, the approaches do not characterize the phenomenon itself. Furthermore, recalibration is still required, if the calibration data-set does not capture all variation appearing the samples for prediction.

In the present study, cherries were chosen as commercially interesting samples. New cultivars with enhanced fruit size and appealing colouration have been released, which are sold at extremely high prices. Such new high-value cultivars raise questions regarding the processes of optimum harvest date determination, selective harvesting, and postharvest handling -

questions that might be answered by means of non-invasive analyses in the supply chain, when a feasible method becomes available.

The approach carried out, was aimed at improving the robustness of VIS spectroscopy by means of sensor fusion approach. The influences of varying optical fruit properties on the pigment calibration were corrected (i) with the effective pathlength in the fruit tissue obtained with DTOF readings as well as (ii) with additional data on the boundary conditions of the sample obtained by means of TIRF analysis. In comparison, commonly applied data processing methods were tested such as (iii) normalized index, (iv) multivariate spectra processing.

For testing the approach, data set for calibration and slightly different data set for validation in similar ranges as it appears in practice were required. The cherries subjected to the experiments were harvested on the same trees, but in earlier and more advanced ripeness stages. Such conditions would occur for all non-destructive equipment applied in the field.

3.3.4. Materials and methods

Fruit material and quality analysis

Sweet cherry, *Prunus avium* 'Schneiders späte Knorpel', was analysed immediately after harvesting in different ripeness stages: unripe, intermediates 1–3 (im1–3), and ripe. Fruits were subjected to chemical pigment analysis as well as VIS/NIR spectroscopy, DTOF, and TIRF readings. Forty-five fruits were harvested on one day at five ripeness stages ($n = 9$) and analyzed within 48 h. Acidity and fruit size were measured.

The anthocyanins and carotenoids contents of the exocarp and mesocarp of cherry were analysed wet-chemically by spectrophotometry after acetone/diethyl ether extraction. In the ether fraction the carotenoids were analysed, while the anthocyanins accumulated in the polar phase. Standards of LUT, aCAR, and bCAR were applied for carotenoids analyses and the anthocyanins were expressed as cyanidin (Carl Roth GmbH, Germany) equivalents, each pigment by subjecting the spectra of extracts to MLR based on the standard solutions and minimising the error when recalculating the sum spectra of extracts (Pflanz and Zude, 2008).

Non-destructive sensing

Colorimetric readings (CM-2600d/2500d, Minolta, Japan) were recorded by means of *L*a*b** (D65) values that were calculated with the instrument software according to CIE standards.

The fruit spectra were recorded (400-850 nm) in diffuse reflectance mode by means of a scanning dual beam spectrophotometer unit (Lambda 950, Perkin Elmer, USA) on referenced

(100 % Spectralon, Labsphere Ltd., North Sutton, U.S.A.) wavelength intensities.

The red-edge, IP of chlorophyll absorption peak determined by f''(660-710 nm) = 0, and a normalised index, $(I_{620} - I_{780})/(I_{620} + I_{780})$, were calculated on the spectral intensities, $I_R(\lambda)$ from VIS/NIR readings. The red-edge is accepted to correct for scattering and resulting vertical shifts of the spectra due to the calculation on the spectra derivatives. The index was calculated on the edge of anthocyanidin absorption passband and a passband in the "optical window" to correct for varying scattering properties.

A system to measure DTOF in transmittance geometry was used, working at 780 and 670 nm, based on a pulsed laser diode (PDL800, PicoQuant GmbH, Germany) with 80 MHz repetition frequency, 100 ps duration, and 1 mW average power, with a compact photomultiplier (R5900U-L16, Hamamatsu Photonics, Japan) and an integrated PC board for time-correlated single photon counting (SPC130, Becker&Hickl GmbH, Germany). The width of the instrumental response function (IRF) was <160 ps (Torricelli, 2009). Acquisition time was set to 1 s per fruit. The values of reduced scattering coefficient and absorption coefficient were obtained from fitting the experimental data with a standard solution of the diffusion approximation to the transport equation for a semi-infinite homogeneous medium. The diffusion coefficient was taken to be independent of the absorption properties of the medium. The theoretical curve was convoluted with the instrumental response function, and was normalised to the area of the experimental curve (Cubeddu et al., 1999). The procedure was described in detail in former studies about fresh horticultural products (Cubeddu et al., 1999; Torricelli, 2009).

TIRF measurements were performed using a confocal imaging system (Infinty 3, Visitron Systems, Germany) equipped with an additional TIRF port. For excitation, the krypton laser line at 647 nm of an argon-krypton ion laser (Innova 90, Coherent, CA, USA) was applied. The excitation light was coupled into the intact cherry sample through a 100x oil immersion objective. The incident angle for determination of the threshold angle for total reflection could be adjusted with a micrometer screw. The threshold position of the screw and related incident angle were calibrated for the refractive index with sucrose solutions of known refractive index containing $\sim 10^{-5}$ M Cy5 (Cy5 excitation and emission maximum at 649 and 670 nm, respectively, quantum yield is 0.28). The refractive index of the calibration solutions was checked with a standard laboratory refractometer (Carl Zeiss, Germany). Calibration, $y = 707.65x - 632.58$, r^2 = 0.99, on the apparent refractive index measured in sucrose solutions (0-18.2 %Brix). The calibration was tested on intact cherries. The prediction referenced by means of destructive refractometric readings of the squeezed fruit juice was r^2 = 0.77.

Calculation of effective pathlengths

The pathlength, L, was determined by the penetration depth, δ, (Equation 3.15) calculated based on diffusion theory (Torricelli, 2009), which resulted in an average value of 0.3 cm

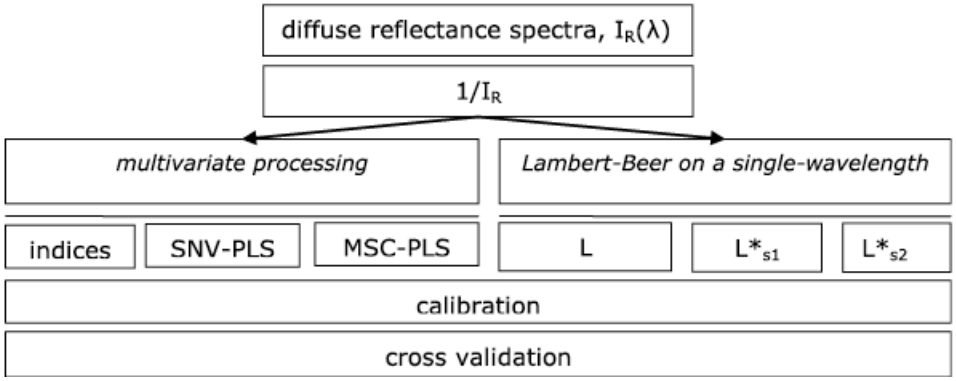


Figure 3.8.: Overview on the calibration steps carried out for non-destructive pigment analysis in cherry. Calibration was applied subsequently on a new test set for validating the approaches (Tables 3.13 and 3.14).

$$\delta(780\,nm) = 1/\mu_{att} = 1/\sqrt{3\mu_a(\mu_a + \mu'_s)}. \tag{3.15}$$

The effective pathlength, $L * s_1$ was calculated by equation (3.16), where c is the speed of light in vacuum ($c \approx$0.03 cm ps^{-1}), and N is the refractive index of the medium. For calculating $L * s_1$ the refractive index was assumed constant with $N = 1.4$, while for calculating $L * s_2$ (Equation 3.17) the actual refractive index was used based on the TIRF readings

$$L * s_1(780\,nm) =< t > c/1.4 \tag{3.16}$$

$$L * s_2(780\,nm) =< t > c/N. \tag{3.17}$$

Calibrations

For calibration and validation (Figure 3.8) the data sets were split in two blocks, one block providing the values for unripe and half of the intermediate samples and the second block containing the values of the remaining intermediates and ripe fruits.

Calibrations were carried out by means of linear regression either using the colour values, index, and results from the application of Lambert–Beer law, using the three pathlengths or by means of multivariate PLS calibration models. The latter were built in Matlab (version 7.6.0.324 R2008a, The MathWorks, USA) on raw data of the VIS/NIR spectra pre-processed by means of MSC and second derivative. The optimum numbers of LV were selected on the basis of minimizing the $rmse$ by selecting the first minimum in cross-validation with leave-one-out routine.

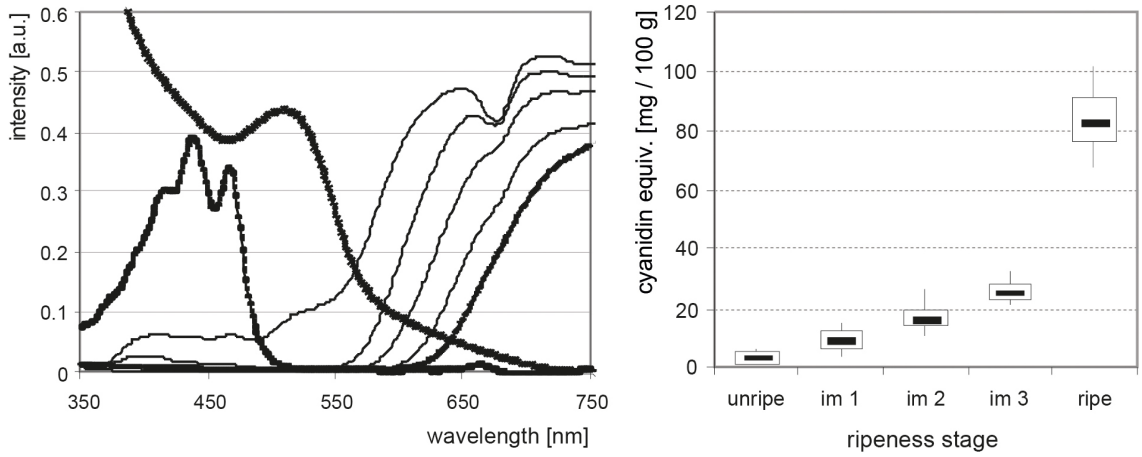
The measuring uncertainty was separated into two independent estimation errors: bias and remaining $rmse$. Percentage estimation errors were obtained by dividing by the mean of actual value. The errors, correlation coefficient (r) and coefficient of determination (r^2) were calculated in Matlab.

3.3.5. Results and discussion

Fruit development and fruit spectra

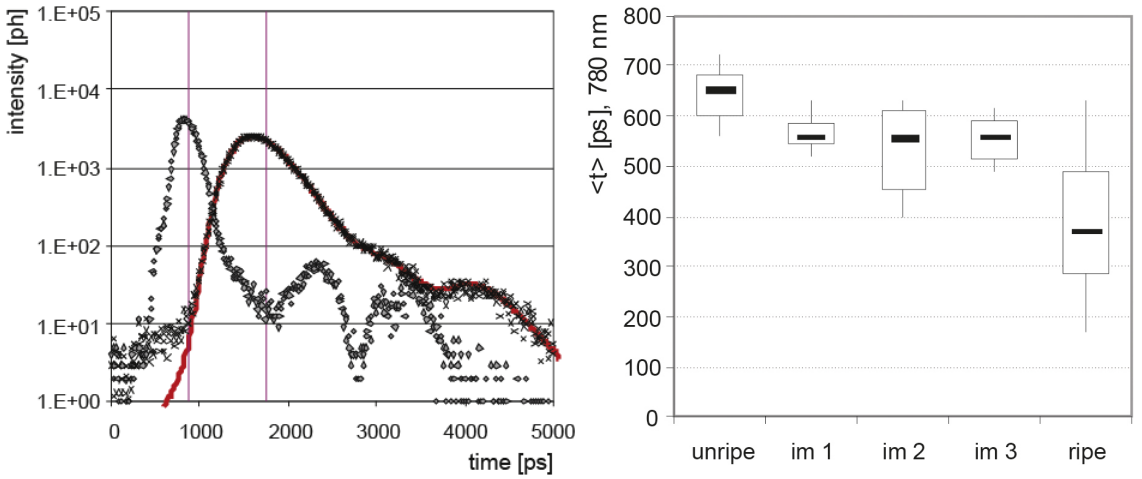
In unripe cherries and fruits in slightly enhanced ripeness stage (im1), chlorophyll was noticed in the fruit spectra around 680 nm, but was at the detection limit in the chemical analyses (Figure 3.9a). The red-edge (660-710 nm), applied as chlorophyll estimator in precision agriculture (Richardson et al., 2002) and fruit analysis (Zude, 2003), indicated decreasing chlorophyll contents providing interesting data on the fruit development. However, in more advanced ripeness stages the chlorophyll already disappeared.

Cherries of all five ripeness stages showed spectral variation at the blue-green passband with a sharp intensity increase appearing in the range of 550-650 nm (Figure 3.9a). The region of enhanced reflectance intensities was shifted further in the red wavelength range the more red pigmentation occurred. Consequently, the intensity change provides a rough marker for the fruit ripeness stage. The edge appearing at the passband of 620 nm was used for further data processing. The non-invasively measurable data are already of practical interest for the production and harvest manager in an orchard, since fruits at advanced ripeness stage exhibiting more intense pigmentation can be selected. In practice, the variation of fruits appearing at the same time on the tree point to the potential value-adding by means of selective harvesting such as carried out in the apple production. Such suggestion appears reasonable since the market price increases exponentially mainly with the ripeness stage and quality regarding the fruit sweetness expressed as SSC and each mm in fruit size (Table 3.11). Resulting, non-invasive readings can help to make the processes, for instance the harvest management, more efficient.



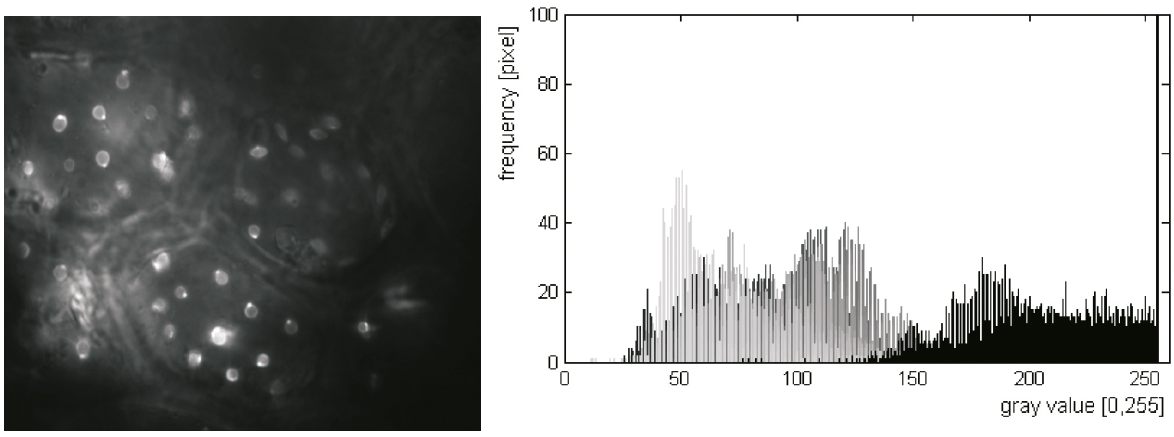
(a) (b)

Figure 3.9.: (a) Mean diffuse reflectance VIS spectra of unripe (bold line), intermediates (line), and ripe (cross) cherries measured in reflectance mode as well as transmittance spectra of unpolar (closed circle) cherry extract containing carotenoids and very small amounts of chlorophylls as well as polar (open circle) fruit extract containing anthocyanidins. (b) Boxwhisker plot with average shown as line symbol of the cyanidin contents in fw of fruits at five ripeness stages.



(a) (b)

Figure 3.10.: (a) Typical instrument response function, IRF, (diamond), and distribution of time of flight, DTOF, readings (cross) at 780 nm, and curve fit (line) according to diffusion theory when measuring cherries are presented. Time position of $< t >$ is marked. (b) Box-whisker-plot shows the time of flight,$< t >$, for the five ripeness stages (unripe, intermediates im1–3, ripe) studied.



(a) (b)

Figure 3.11.: Fluorescence image from cherry fruit obtained in the evanescent field with excitation at 647 nm (a) and regression of angle-dependent appearance of evanescent field (D) with histogram of image obtained in total reflectance (gray columns) and outside evanescent field (black columns) presented in one diagram (b).

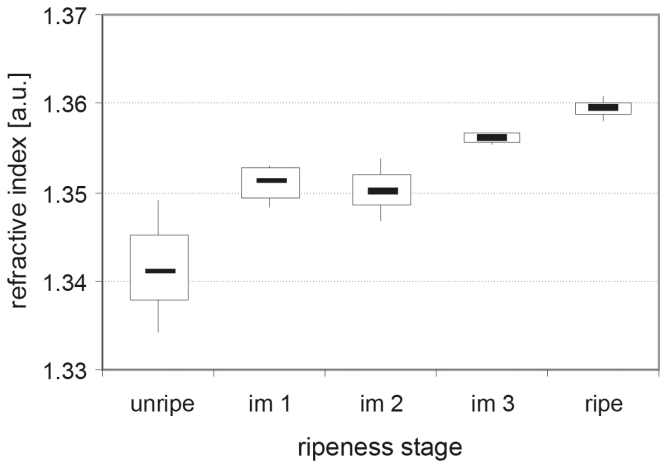


Figure 3.12.: Refractive index of sweet cherry measured by means of TIRF readings.

Table 3.11.: Correlation table of fruit data obtained on five ripeness stages ($n = 43$) of sweet cherry.

	Size	SSC	Acidity	CAR_{total}	Cyanidin	Rededge	*L**	*a**	*b**	$< t >$	*N*
Size	1.00	0.02	0.30	0.07	0.28	0.17	-0.05	0.00	-0.07	0.38	0.14
SSC		1.00	-0.22	-0.12	0.76	-0.84	-0.68	-0.69	-0.74	-0.46	0.74
Acidity			1.00	0.61	-0.55	0.48	0.40	0.57	0.53	0.65	-0.32
CAR_{total}				1.00	-0.34	0.32	0.18	0.34	0.30	0.53	0.02
Cyanidin					1.00	-0.96	-0.53	-0.75	-0.69	-0.64	0.68
Rededge						1.00	0.54	0.74	0.69	0.64	-0.73
*L**							1.00	0.66	0.90	0.50	-0.78
*a**								1.00	0.91	0.54	-0.52
*b**									1.00	0.56	-0.70
$< t >$										1.00	-0.44

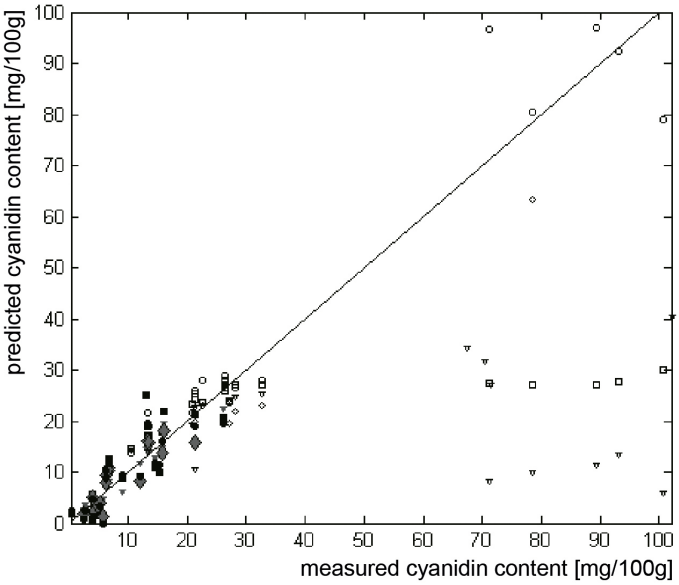


Figure 3.13.: Scatter plot of measured and predicted values of cyanidin contents in fw of cherry fruit in the calibration (closed symbols) and in the independent test-set validation (open symbols) on fruits in advanced ripeness stages by means of data analyses based on $L * s_1$ (circle), $L * s_2$ (diamond), and constant $L = 0.3$ (square), as well as frequently applied PLS with MSC pretreatment (triangle).

Table 3.12.: One-way analysis of variance (ANOVA) and post hoc Tukey test was applied for grouping. Values are given as mean ± SE, while mean separation within a row (a–e) indicates significant differences at 0.05.

	Unripe	im1	im2	im3	Ripe
Cyanidin	3.25 ± 1.89a	9.03 ± 4.02a	15.9 ± 4.88a	32.74 ± 24.12b	80.44 ± 12.8c
Log I_{620}	0.2010 ± 0.05a	0.5275 ± 0.08b	0.9491 ± 0.13c	1.2167 ± 0.09d	1.2849 ± 0.03ed
$< t >$	651.82 ± 55.60a	556.72 ± 37.90a	553.05 ± 77.99a	525.57 ± 109.61a	388.96 ± 222.89b
$L * s_1$	13.97 ± 1.19a	11.93 ± 0.81a	11.85 ± 1.67a	11.26 ± 2.34a	8.33 ± 4.78ba
N	1.3412 ± 0.010a	1.3514 ± 0.002b	1.3501 ± 0.003b	1.3562 ± 0.000bc	1.3598 ± 0.001dc
$L * s_2$	15.04 ± 1.13a	12.54 ± 0.99a	12.23 ± 1.88a	12.19 ± 0.74a	11.41 ± 3.94a

Table 3.13.: Statistics of calibration and validation results, in the latter, using an independent test-set capturing fruits in advanced ripeness stages. Non-destructive analysis of fruit cyanidin contents was carried out using linear regression by means of colour data, *L*a*b**, and wavelength-specific normalised index, $(I_{620} - I_{780})/(I_{620} + I_{780})$.

	Index	*L**	*a**	*b**
	$y = 0.0268x + 0.358$	$y = -1.2494x + 45.551$	$y = -1.1681x + 37.546$	$y = -0.8665x + 21.198$
Calibration results on unripe and intermediates 1 and 2 ($n = 23$)				
% $rmse$	50.927	63.323	108.857	34.950
%bias	-25.454	0.000	0.001	-10.981
r^2	0.581	0.517	0.270	0.592
Validation uncertainty on intermediate stage 3 and ripe cherries (n =20)				
% $rmse$	2.065	6.254	56.711	7.262
%bias	-152.888	-133.714	46.474	-112.601
r^2	0.246	0.363	0.670	0.600

Table 3.14.: Measuring uncertainty of non-destructive analysis of fruit cyanidin contents, with validation on fruits in advanced ripeness stages, by means of Lambert–Beer law applying different degree of sensor fusion and resulting pathlengths: $L = 0.3$, $L * s_1$, $L * s_2$, as well as from whole spectra PLS regression analysis.

L = constant	$L * s_1$	$L * s_2$	PLS-model on data pretreated with MSC using 3 LV	PLS-model on 2nd derivative using 4 LV
$y = 0.0035x + 0.0097$	$y = 0.0028x + 0.0088$	$y = 0.0044x + 0.0035$		
Calibration on unripe and intermediates 1 and 2 ($n = 23$)				
% $rmse$	36.909	39.894	28.702	28.420	14.045
%bias	-0.530	0.619	-0.904	6.024	8.413
r^2	0.667	0.669	0.707	0.767	0.985
Validation result on intermediate stage 3 and ripe cherries (n =20)				
% $rmse$	12.640	10.626	3.053	6.036	16.617
%bias	-33.176	4.919	-8.108	-138.435	-113.359
r^2	0.453	0.921	0.950	0.142	0.004

In cherry, the red pigmentation is caused by the anthocyanins and carotenoids contents. Even if the cumulative increase of red pigments provides some information, a more specific data analysis of the fruit spectra would be helpful for characterizing the fruit development onsite by means of non-invasive readings. Comparing the evolution of red pigment contents, the aCAR decreased, while the CAR_{total} did not show consistent differences during fruit development. The anthocyanin – here expressed as cyanidin equivalents – content was enhanced with higher ripeness stages (Figure 3.9b). Although the aCAR and the anthocyanins changed significantly with the fruit development, only the anthocyanins were studied further due to their higher contents and, therefore, more pronounced contribution to the changes in the fruit spectra.

Calculation of the effective pathlength

A robust and non-invasive analysis of the pigments was approached for characterizing the fruit ripeness stage. The pigment analysis was carried out by applying Lambert-Beer's law directly on the intensity data, I_R(620 nm) obtained with VIS/NIR remittance readings and the constant pathlength, $L = 0.3$ cm. The constant pathlength (Equation 3.15) was derived from diffusion theory, which is obviously underestimating the apparent light penetration. The suitability of different theories and optical geometries (Arridge et al., 1992) has been explored by different work groups. However, the diffusion theory is widely used and applied in the present work for principally testing our approach.

Additionally, the effective pathlength was estimated by means of DTOF. The mean time-of-flight ($< t >$) was derived from the distribution of incoming photons recorded (Figure 3.10a). The DTOF was measured for wavelength $\lambda = 780$ and $\lambda = 670$ nm. The readings from 670 nm gave a weak signal in ripe cherries due to high absorption coefficients of cyanidins in more advanced ripeness stages. Resulting, the data of 780 nm (Figure 3.10b) were used further for calculating the effective pathlength, $L * s$ (Equations 3.16 and 3.17).

For biological tissue $N = 1.4$ is typically used as an estimation. However, the cherries developed enhanced values of N and corresponding SSC during fruit ripening – the latter ranging from 9.9 to 20.1 °Brix. Since a possible influence on the calculation should be neglected in the present study, the actual refractive index was measured non-destructively by means of TIRF. Structures from cell compounds appeared in the image (Figure 3.11a), when the fluorescence appeared in the evanescent field and total internal reflection occurred. According to Snell's law, the refractive index was derived. The angle of total reflectance was sensitively recorded by means of histograms of the images (Figure 3.11b). At the threshold angle the excitation light no longer coupled into the sample via evanescent field, but directly, leading to a dramatic increase in intensity und thus sudden light overexposure of the image.

Based on the calibration with standard solution, the refractive index at wavelength 647 nm was non-destructively measured. The N changed over the different ripeness stages (Figure 3.12), and was tested in calculating the effective pathlength, $L * s_2$ (Equation 3.17).

Resulting, in the present study, the constant value of $N = 1.4$ for calculating the effective pathlength, $L * s_1$, and the data obtained by means of non-invasive TIRF for calculating the effective pathlength, $L * s_2$, were applied. Accordingly to the change in refractive index, the effective pathlength changed, resulting in 9.64 % higher values when applying the refractive index estimated from TIRF readings (Table 3.12).

Calibration on cyanidin contents based on Lambert-Beer

Simple linear calibrations on the cyanidin contents of cherry fruit were carried out based on colour data and the use of a normalised index, calculated on the absorption maxima of pigments and a passband in the optical window to correct for varying scattering properties. Calibration results point to expected highest correlation of *b**-value and cherry cyanidin contents (Table 3.13).

Loadings of PLS regression analyses showed highest variance of first three and four LVs around 624 nm passband supporting the choice of I_R(620 nm) for calculations. At three LVs the minimum in cross-validation in leave-one-out mode was reached, capturing variances of 98.69 % and 82.53 % in spectral intensity and cyanidin content, respectively. Increased r^2-value was found when calculating on $2nd$ derivative obtained by means of Savitzky–Golay routine with window size of 5 wavelengths and $2nd$ order polynom (Table 3.14).

Furthermore, the constant as well as effective pathlengths were subjected to the pigment, c_i, analyses by means of Lambert-Beer with: $I_R(620\,\text{nm}) = c_i L * s_n(780\,\text{nm})$. The calibration step was carried out on unripe and intermediate (im1–2) cherry fruits. The constant pathlength as well as the effective pathlength, obtained by means of DTOF, assuming a refractive index of 1.4, and the effective pathlength, calculated by DTOF taking into account the actual refractive index obtained from TIRF readings, resulted in low bias but rather high diffusion errors in all calibrations (Table 3.14). In former studies (Zude et al., 2008b) the method of fusing VIS spectra and DTOF data was tested for liquid phantoms mimicking the optical properties of fresh carrot. Lambert–Beer law using a constant pathlength as well as combined application of the intensity at a specific wavelength and the effective pathlength had been resulted in low calibration errors with $r^2 > 0.98$. However, when the two calibrations were applied on phantoms mimicking changes in the scattering properties, validation results of $r^2 = 0.47$ and $r^2 = 0.64$ with reduced bias but also high diffusion error were found, respectively.

Validation results

Interesting results were found when using the calibration models on samples in ripe and overripe stages - providing variation in the scattering coefficient and resulting effective pathlength (Figure 3.10b). The validation point to an increase in r^2 and obvious bias reduction

(Figure 3.13) when using the effective pathlength data (Tables 3.13 and 3.14). The high coefficients of determination when using the apparent effective pathlengths partly appear due to extremely high variation in the cyanidin contents, exceeding the range in the calibration.

Coefficients of determination in the validation were $r^2 = 0.45$ and $r^2 = 0.92$, comparing the use of spectral intensities from VIS/NIR readings or combined use of spectral intensities and effective pathlength from DTOF analysis, respectively (Table 3.14).

Validation based on normalized index gave r^2-value of 0.25; colour statistics remained rather stable, while PLS models resulted in dramatically decreased $r^2 = 0.14$. In the validation, the errors appearing when PLS regression analysis on pretreated spectral data was used, can certainly be reduced by means of larger data set used in the calibration and further improved by means of different data pre-processing, or by using MLR. When using the entire data set including unripe to overripe cherries in the calibration with MSC pre-treatment, the statistical results of cross validation in leave-one-out mode were: $r^2 = 0.83$, $rmse = 20.58\,\%$, and bias was - 14.26 %.

However, big data sets capturing all possible changes in the optical properties of a fruit under question are necessary for PLS calibration, but are rarely available in practice. Fruits are changing not only with the ripeness stage, but also from year to year, during tree ontogenesis, according to the production system, and environmental conditions. Consequently, the measurement of the effective pathlength provides an interesting alternative for developing robust calibrations instead of extremely large data sets for multivariate calibration with statistical pre-treatment.

3.3.6. Conclusions

It was pointed out that (i) the effective pathlength of photons traveling in the fruit is one main influencing factor on the robustness of calibrations, and (ii) it is possible to correct for this varying value. Such conclusion can be assumed, even if the results on the reduced scattering coefficient and the effective pathlength calculated can be seen only as principle proof due to inaccuracy of diffusion theory in modeling highly absorbing tissue, use of the reduced scattering coefficient that does not provide distinct data on g and μ_s, small fruit samples which cannot assumed to be infinite, and a marginal wavelength dependence of μ_s' in the relevant wavelengths (780 and 620 nm).

Particularly in fresh, rapidly developing produce with changes in the chemical composition as well as texture, the effective pathlength can be used to correct for variation in the scattering coefficient that disturbs the apparent signal of non-destructive analyses in the field.

Acknowledgment

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4. Conclusions

In the context of variable pigment compositions in fresh fruit and vegetables, the present work evaluates non-destructive optical measurements on intact plant material and fruit extracts. Through a review on literature this thesis points to the impact of production and postharvest conditions on the fruit and vegetable pigment contents (Table 1.1, A.1 and A.2). These studies moreover suggest the potential of using the plant pigments to determine the optimal harvest date or a product's shelf life at controlled storage conditions. Particularly, a continuous monitoring of individual chlorophylls and carotenoids should provide precise data on physiological stages of fruit on the plant or in postharvest.

That is reasonable, due to a degradation of chlorophylls that occurs concomitantly with an enhanced carotenogenesis during the fruit development. In particular it leads to a complex pattern of individual carotenoids (Goodwin and Jamikorn, 1952; Mackinney and Jenkins, 1952; Cabibel and Ferry, 1980; Gross, 1987). After the conversion of chloroplasts into chromoplasts, different carotenoids appear and cause the yellow and red colours, which is consequently the human perception of interfering light absorption in ripening fruit. This was apparent in the experiments by means of spectral readings, analysing the carotenes and xanthophylls qualitatively and quantitatively (Pflanz and Zude, 2008; Pflanz et al., 2010).

Carotenoids absorb light wavelength-selectively from the UV part of the electromagnetic spectrum, chlorophylls and anthocyanidins mainly from the VIS range. Regarding this spectral differentiation, a few devices have been developed to assess variable plant compounds by non-destructive UV/VIS spectroscopy (see chapter 2). However, the coexistence of multiple chromophors in biological tissues of vegetables and fruit leads to perturbations of spectral readings through masking light absorption and scattering. But even if Lambert-Beer's law is valid due to a lack of scattering in dissolved pigment mixtures, spectral masking of carotenoids occurs as well. Often then absorption maxima of each compound are used for simultaneous calculation of individual pigment concentrations. This could be erratic for separating coinciding absorption if two pigments have almost identical absorption coefficients at the same wavelength range (e.g. LUT and aCAR). Moreover, such calculations are not suitable for a qualitative separation of different compounds.

For this reason, the developed iterative multiple linear regression aimed at a quantitative and qualitative separation of coinciding signal readings in the UV/VIS through spectral profiles of high resolution from individual plant pigments. Similar approaches have been used to dismantle spectra of inorganic and organic acids (Herschberg, 1964), as well as absorption

spectra of CHLa and CHLb (Marr et al., 1995). Beyond such analysis of individual chlorophylls, the iMLR is being extended to analyse carotenes and xanthophylls, which typically occur in horticultural products.

In regard to tomato pigments, the present study points to the fact that no specified equations for LUT determination have been cited in literature yet. Nevertheless, it should also be possible to use some of the given equations for aCAR determination in analysing tomato extracts, because the different spectral profiles of LUT and aCAR are corresponding at 445 nm. This has been shown for the determination of aCAR, bCAR, and LYC from pigment extracts of lycopene-containing carrots (Nagata, 2007). However, immature tomatoes contain high contents of chlorophylls, which do not occur in carrots. Thus, Nagata and Yamashita's equation can be recommended to a certain extend for use in tomato analyses disclosing the degradation of chlorophyll. It can only be applied on fruits which have already changed their colour from green to orange. In ripening tomato fruit the contents of bCAR determined by using iMLR are comparable to those shown in the literature. The high measuring uncertainty when using established equations has been proved in the present work. Limitations are not present in the iMLR approach, where chlorophylls are separated in advance of the carotenoids. The sum signal of carotenoid absorption at the UV wavelength range is being corrected through the previously fitted chlorophyll signatures. Thus the iMLR can also be applied for chlorophyll-containing pigment compositions.

In comparison with commonly used equations, variable contents of LUT were only detected through the new approach. It should also be pointed out that if LUT have not been taken into account for the analysis of LUT-containing pigment mixtures, the content of bCAR could be over-represented by using simple equations. The coincided absorption of LUT and bCAR, typical in fresh tomato and fruit extracts, leads to erratic results if the analysis is performed by equations, which consider bCAR and LYC only. This is, e.g., relevant for the analysis of tropical fruit containing individual carotenes and xanthophylls such as in mango or papaya.

According to the present studies on intact fruit, in-situ spectral readings have been carried out by means of spectrophotometry in remission geometry. In this regard photon's pathway through the biological tissue is crossing deeper cell layers in comparison to diffuse reflectance readings (that includes colour charts, CIELAB colorimeters or even spectral readings in reflection mode). While the colour change of tomato fruit is reasonably closely correlated with variable contents of carotenoids, in mango fruit the determination of ripeness using OECD-colour charts or instrumental colour measurements is not recommended. The content of skin chlorophylls is cultivar-dependent, distributed with a typical spatial pattern, and thus, the light absorption from carotenoids located in deeper layers is visually masked by chlorophyll absorption. This was apparent in the present study on mango fruit. Within the sample set with high variability at the ripeness stage, the skin colour was less closely correlated with chemically determined chlorophyll contents in comparison to remittance readings.

In cherry fruit, chlorophylls were almost degraded at medium ripeness stages and have thus

minor relevance for quality and shelf-life estimation. According to optical readings the contents of anthocyanins and carotenoids contain potentially more information for e.g. harvest management. However, the amount of anthocyanins is cultivar-, UV- and temperature-dependent and moreover their absorbance passband ranges widely from 460 up to 550 nm, which leads to significant masking effects of carotenoids. Consequently, also here the new approach of iMLR can potentially improve the separation of such coincided spectra. Influences from scattering effects on *in-situ* measured spectra have also been figured out in the study on cherry fruit (Zude et al., 2011). To compensate for varying scattering coefficients, the iMLR approach was helpful for developing robust calibrations for *in-situ* analyses of fresh fruits. Considering this correction of the scattering effects, in the future, robust calibrations for fruit from different cultivars and varying development stages might be possible. The correction was carried out by means of the effective pathlength of photons. Consequently, it seems reasonable to combine the effective pathlength data with the results of iMLR by parameter fusion to get more stable models of plants' physiological stages in terms of phytomonitoring applications.

In conclusion, the iterative algorithm is applicable for laboratory analyses calculating individual chlorophylls, carotenes and xanthophylls in plant extracts. It increases the efficiency of spectral analyses, both in terms of time requirements as well as costs for chemical preparation decline. As a part of the methodology in future applications, it could make non-destructive UV/VIS spectroscopy more feasible for sustained fruit monitoring in the context of precise horticultural cultivation. The approach as described in this work can help to reduce product quality losses along the whole supply chain of horticultural products. While the variable scatter properties of biological tissue are still an issue, commercial devices for time-resolved (or spatially-resolved) spectroscopy may solve this problem. In general, the separation of absorption and scatter effects may lead to novel and better calibration models for quality-related attributes. That could make horticultural processes more efficient by keeping costs moderate and saving resources with regard to optimal cultivation conditions – such as harvest date and postharvest storage.

Furthermore, the availability of affordable optical instruments provides many opportunities for quality evaluations of horticultural products. Mobile or even handheld spectrophotometers are already commercially available and not only used in scientific studies. According to the persistent miniaturisation of electronic devices and declining prices, it can be assumed that compact spectrophotometers will come in similar sizes to small data loggers for temperature or relative humidity. This would give access to direct measures on the plant or within the process chain in storage and transport carriers.

In a modern economy of short-term demands, growers need differentiated information on the cultivated crops that make delivery more adaptable to the market. Here, the analyses of timeseries based on optical readings on the intact produce could provide data on the constantly changing properties of fresh fruit and vegetables. This was apparent in a postharvest study on papaya fruit, showing the potential of non-destructive spectroscopy linked with kinetic analysis of pigments during fruit development (unpublished results). Otherwise, the

development of autonomous harvesters equipped with sensors is now in progress and will become more important. However, from the author's point of view, this technology is not yet sophisticated enough for practical use under horticultural conditions. Indeed, intelligent optical sensor technologies might be part of smart spectacles (augmented reality) used by human harvesters. In doing so, the ripeness stage of fruit or vegetables would be displayed immediately as being ready to harvest or not. Simultaneously, the crop attributes will be stored in relation to spatial positions in a geo-referenced database of the entire orchard. This could make the harvest more efficient through coordinated scheduling of employees. A mapping of the fruit development by means of individual contents of carotenoids and chlorophyll could further help to disclose issues of cultivation strategies. Such projects are presently supported by the European Commission.

However, due to new cultivars, recent studies on fruit quality and maturity are not always comparable to those shown in the literature. Many studies were carried out on cultivars that are not grown nowadays. In addition, sometimes the environmental conditions showing an impact on the content of pigments were not specified closely enough. But whereas total contents of chlorophylls and carotenoids could be interrelated, it is recommended to determine individual pigment contents for a more differentiated insight into the physiological crop development. Individual pigment changes should consequently be introduced as indices of maturity and quality.

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A. Tables

Table A.1.: Preliminary list of studies with reference to *preharvest* environmental parameters and varying pigments in horticultural products. Pigments of apple fruit were not investigated in this thesis, but shown due to their scientific and economic relevance.

	Crop	Cultivar	Pigment (trend)	Non-destructive analysis	Reference method	Remarks	References
Temperature	Tomato	Early Red Chief	LYC (+)	Hunter LAB	TLC	efficient biosynthesis between 10-30°C	(Koskitalo and Ormrod, 1972)
		Counter	LYC (+)	Hunter LAB	HPLC	efficient biosynthesis between 18-23°C	(Krumbein et al., 2006)
		n.s.	LYC (-)	Colour appearence	LC	inhibition above 30°C	(Duggar, 1913; von Euler et al., 1931)
	Apple	Fuji Jonagold Jonathan	ANT_{total} (+)	–	LC	at low temperture	(Arakawa, 1991)
Radiation	Tomato	Apricot genotype Summer Sunrise	bCAR (+) LYC (o)	–	TLC	light stimulated CAR_{total} biosynthesis	(Raymundo et al., 1976)
		Illinois T19	CAR_{total} (+) LYC (+)	–	LC	illumination of sunlight	(McCollum, 1954)
	Mango	Nam Dok Mai #4	bCAR (+)	CIELAB	LC	brighter yellow peel at UV hidden treatments	(Chonhencho et al., 2011)
	Apple	Granny Smith	CAR_{total} (-)	Spectrophotometry	TLC	adaption to high solar radiation	(Merzlyak and Solovchenko, 2002)
		Antonovka	CAR_{total} (+)	Spectrophotometry	TLC	short-time treatments of UV irradiation	(Merzlyak and Solovchenko, 2002)
		Akane Sansa	ANT_{total} (+)	–	HPLC	high intensity artificial illumination	(Ubi et al., 2006)
		Fuji Pinova	ANT_{total} (+)	CIELAB	–	reduced sunlight due to hailnet shading	(Solomakhin and Blanke, 2010)
Atmosphere	Tomato	Counter	bCAR (o) LYC (o)	Hunter LAB	TLC	varying levels of CO_2 in greenhouse atmosphere	(Krumbein et al., 2006)
		Pannovy	bCAR (+) LYC (+)	Colour chart	LC	high level of CO_2 and relative humidity	(Dannehl et al., 2012)
	Mango	Tommy Atkins Kent	CAR_{total} (++)	Colour chart	–	normal atmosphere and room temperature	(Lizana and Ochagavia, 1997)
Soil	Tomato	n.s.	CAR_{total} (+)	Hunter LAB	LC	moderate adjusted salinity	(Smirnoff, 1995; Dorais et al., 2001)
		Durinta	CAR_{total} (o)	CIELAB	LC	varying soil salinity	(Petersen et al., 1998; Krauss et al., 2006)
		n.s.	LYC (-)	–	TLC	low levels of moisture stress	(Dorais, 2007)
		Yellow Carol	CAR_{total} (-)	CIELAB	HPLC	high water supply (higher water content in fruits)	(Matsuzoe et al., 1998)
		Daniela F1	LYC (+)	–	LC	low water supply	(Brandt et al., 2003)
Nutrient supply	Tomato	n.s.	bCAR (+) LYC (+)	–	HPLC	limited nitrogen supply	(Trudel and Ozbun, 1971)
		Izabella	bCAR (+) LYC (+)	–	HPLC	organic cultivation	(Caris-Veyrat et al., 2004)
		PT 4769	bCAR (o) LYC (o)	CIELAB	HPLC	comparision of organic and conventional cultivation	(Lumpkin, 2005)
		Counter	CAR_{total} (o)	Hunter LAB	TLC	different nutrition treatments	(Krumbein et al., 2006)
		Fireball	CAR_{total} (+) LYC (+)	–	HPLC	higher K-levels	(Trudel and Ozbun, 1970)
	Cherry	Buttner's Red	ANT_{total} (+)	–	HPLC	higher boron fertilisation	(Wojcik and Wojcik, 2006)
	Apple	York Imperial	ANT_{total} (++)	Colour chart	TLC	sprayed boron application	(Dunlap and Thompson, 1959)
		Starking Delicious	ANT_{total} (o)	Hunter LAB	–	application of boron	(Peryea and Drake, 1991)
		Gala	CAR_{total} (+)	CIELAB	LC	urea and magnesium application onto canopy	(Reay et al., 1998)

not specified (n.s.); degradation (-); no significant differences (o); slight increase (+); high accumulation (++)

Table A.2.: Preliminary list of studies with reference to *postharvest* environmental parameters and varying pigments in horticultural products. Pigments of apple fruit were not investigated in this thesis, but shown due to their scientific and economic relevance.

	Crop	Cultivar	Pigment (trend)	Non-destructive analysis	Reference method	Remarks	References
Temperature	Tomato	n.s.	bCAR (o)	Spectrophotometry	–	no inhibition above 30°C	(Vogele, 1937)
		n.s.	LYC (+)	–	LC	slight accumulation at 0°C in storage duration of 12 days	(Goodwin and Jamikorn, 1952)
		n.s.	LYC (-)	–	LC	high inhibition above 30°C	(Goodwin and Jamikorn, 1952)
		n.s.	aCAR (+) bCAR (+)	–	LC	at ranges of 0-30°C and between 6 up to 9 days of storage	(Goodwin and Jamikorn, 1952)
	Mango	Alphonso	bCAR (o) XAN_{total} (o)	Colour chart	LC	limited synthesis stored below 7°C	(Thomas, 1975)
		Alphonso Ataulfo	bCAR (o)	Hunter LAB	HPLC	storage at -40°C	(Cano and Deancos, 1994)
		Kent Keitt	CAR_{total} (-)	Hunter LAB	HPLC	during storage at 5° for 25 days	(Gonzalez-Aguilar et al., 2008)
		Tommy Atkins	bCAR (-), VIO (-)	–	TLC	heat treatments of slices and puree at 80°C for 10 min	(Godoy and Rodriguez-Amaya, 1987)
	Cherry	Lambert Compact	CYA (o) PEL (o) PEO (o)	CIELAB	HPLC	storage at low temperature (2-4°C) for 12 days	(Mozetic et al., 2006)
		Ferrovia Sciazza	CYA equv. (+)	CIELAB	HPLC	storage at 1°C and 94% r.H. for 15 days	(Esti et al., 2002)
		Vittoria	ANT_{total} (-)	Hunter LAB	TLC	85°C water treatment; tissue browning was observed	(Forni et al., 1993)
	Apple	Cox's Orange	bCAR (o) LUT (o) VIO (o)	–	LC	storage at 12°C	(Knee, 1972)
Radiation	Tomato	Liberto	bCAR (+) LYC (+)	–	LC	treatments with UV radiation longer than 22h	(Pèrez et al., 2009)
	Mango	Keitt	CAR_{total} (-)	Hunter LAB	–	in white shaded peel	(Hofman et al., 1997)
	Cherry	Napoleon Satohnishiki Seneca Takasago	ANT_{total} (+)	CIELAB	LC	treatment by means of artificial UV radiation	(Kataoka et al., 2005)
	Apple	Antonovka	ANT_{total} (++)	Spectrophotometry	TLC	apple peel with exposition of high solar irradiation	(Merzlyak and Solovchenko, 2002)
		Jonathan	ANT_{total} (+)	–	LC	after white light treatments	(Arakawa et al., 1985)
Atmosphere	Mango	Kent	CAR_{total} (o)	Hunter LAB	–	delayed carotenoid biosynthesis at adjusted atmosphere	(Trinidad et al., 1997)
		Ataulfo	CAR_{total} (o)	CIELAB	HPLC	storage for 9 days at 5°C	(Gil et al., 2006)
		Siah-e Mashhad	CAR_{total} (o)	–	TLC	22 days at 5% O2 and 10% CO_2	(Khorshidi et al., 2011)
		Tommy Atkins	CAR_{total} (o)	CIELAB	–	high CO_2 concentration in packages	(Rodov et al., 1997)
	Cherry	Burlat	ANT_{total} (-)	CIELAB	LC	stored at less permeable materials	(Remon et al., 2000)
		n.s.	ANT_{total} (-)	–	HPLC	high realtive humidity and microbiological infection	(Macheix et al., 1990)
	Apple	Jonagold, S'ampion	ANT_{total} (-)	–	LC	7 days at 2% CO_2, 2% O_2, 95% r.H.	(Leja et al., 2003)

not specified (n.s.); degradation (-); no significant differences (o); slight increase (+); high accumulation (++)

Table A.3.: Preliminary list of spectral indices which are suitable for non-destructive monitoring of varying pigments in horticultural products.

Mode[a]	Formular	Notation	Corresponding fruit attribute	r^2	Sample	Reference
Diffuse reflectance	$log\frac{R_{800}}{R_{550}}$	n.s.	chlorophylls	0.942	leaves of bean plants (*Phaseolus vulgaris* L., var Fori)	(Buschmann and Nagel, 1993)
	$R_{800} - R_{550}$	n.s.		0.906		
	IP	red-edge		0.929		
	$\frac{R_{800}-R_{680}}{R_{800}+R_{680}}$	NDVI		0.692		
	$\frac{R_{750}}{R_{550}}$	n.s	chlorophylls	0.956	leaves from tobacco plants (*Nicotiana tabacum* L.)	(Lichtenthaler et al., 1996)
	$\frac{R_{750}}{R_{700}}$	n.s.		0.960		
	$\frac{R_{800}-R_{680}}{R_{800}+R_{680}}$	NDVI		0.662		
	$\frac{R_{800}}{R_{700}} - 1$	n.s.	chlorophylls	0.940	apple fruit (*Malus domestica* Borkh. cv. 'Antonovka Obiknovennaia', 'Granny Smith', 'Renet Simirenko' and 'Zhigulevskoe')	(Merzlyak et al., 2003a)
	$\frac{R_{800}}{R_{520}} - \frac{R_{800}}{R_{550}}$	n.s.	bCAR	0.830		
	$\frac{R_{678}-R_{480}}{R_{800}}$	n.s.	bCAR/chlorophylls	0.940		
	$\frac{R_{800}}{R_{550}} - \frac{R_{800}}{R_{700}}$	n.s.	ANT_{total}	0.930		
	$\frac{R_{800}}{R_{678}} - 1$	n.s.	bCAR/chlorophylls	0.950	apple fruit (*Malus domestica* Borkh. cv. 'Antonovka Obiknovennaia')	(Solovchenko et al., 2005)
	$R_{800}(\frac{1}{R_{520}} - \frac{1}{R_{700}})$	n.s.	bCAR	0.880		
	$\frac{R_{620}-R_{780}}{R_{620}+R_{780}}$	NAI	ANT_{total}	0.581	cherry fruit (*Prunus avium* L. cv. 'Schneiders späte Knorpel')	(Zude et al., 2011)
	IP	red-edge		0.960		

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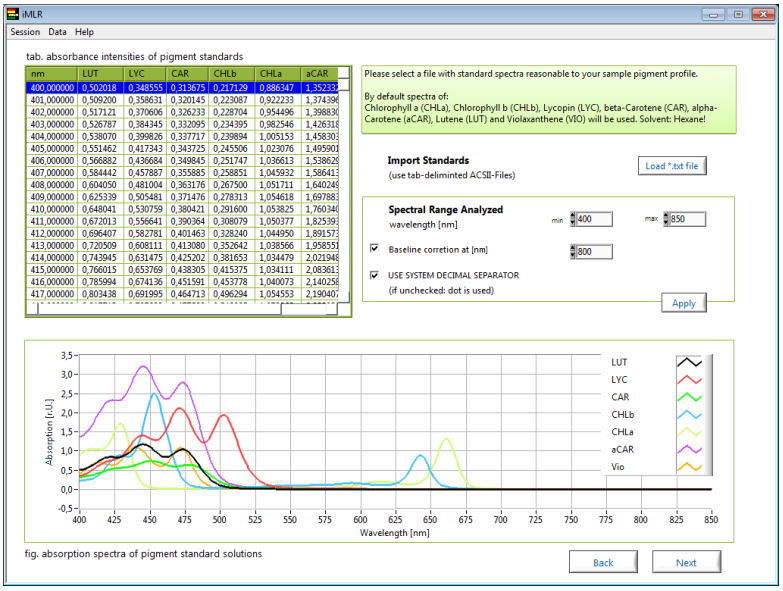
Table A.3 – continued from previous page

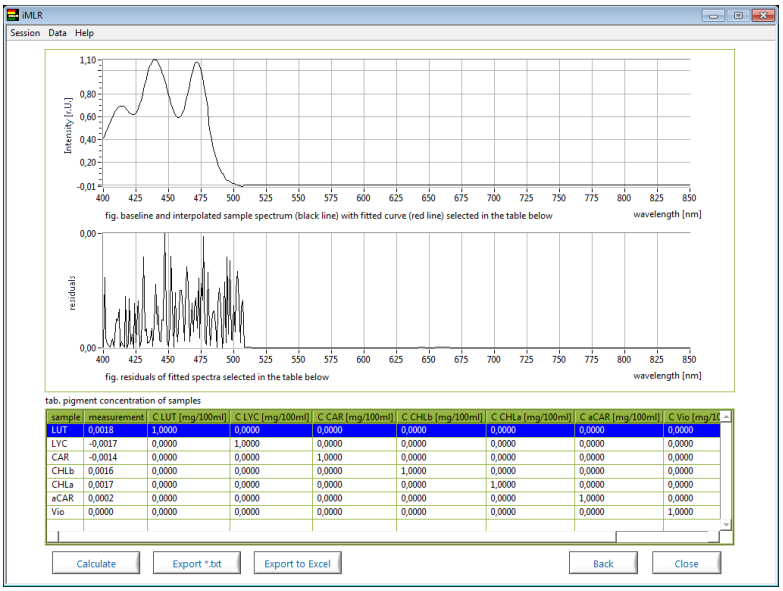
Mode[a]	Formular	Notation	Corresponding fruit attribute	r^2	Sample	Reference
Partial transmittance	$\frac{Tr_{760}-Tr_{695}}{Tr_{760}+Tr_{695}}$ $\frac{Tr_{760}-Tr_{570}}{Tr_{760}+Tr_{570}}$ IP	I_{NChl} I_{NA} red-edge	harvest date	n.s.	apple fruit (*Malus domestica* Borkh. cv. 'Elstar')	(Herold et al., 2005)
	$\frac{Tr_{698}}{Tr_{760}}$	n.s.	chlorophylls	0.879	apple fruit (*Malus domestica* Borkh. cv. 'Elstar')	(Zude, 2003)
	$\frac{Tr_{714}+Tr_{752}}{2} - Tr_{733}$	RVSI		0.686		
	IP	red-edge		0.877		
	$\frac{Tr_{750}-Tr_{705}}{Tr_{750}+Tr_{705}}$	NDVI		0.825		
	$\int_{705}^{750} \frac{Tr_\lambda}{Tr_{705}} - 1\delta\lambda$	n.s.		0.762		
	$\frac{Tr_{698}}{Tr_{760}}$	n.s.	chlorophylls	0.709	apple fruit (*Malus domestica* Borkh. cv. 'Jonagold')	(Zude, 2003)
	$\frac{Tr_{714}+Tr_{752}}{2} - Tr_{733}$	RVSI		0.365		
	IP	red-edge		0.674		
	$\frac{Tr_{750}-Tr_{705}}{Tr_{750}+Tr_{705}}$	NDVI		0.732		
	$\int_{705}^{750} \frac{Tr_\lambda}{Tr_{705}} - 1\delta\lambda$	n.s.		0.345		

[a] Mode of measurement (compare with figure 2.2)

B. iMLR - graphical user interface



(a)



(b)

Figure B.1.: Version 1.55 (2012) of iMLR's graphical user interface. Designed by Michael Pflanz and developed as stand alone application in LabView (Version 6.1, National Instruments Corp., USA) by Christian Regen. (a) Dialog for importing spectral signatures of references and baseline correction (b) Summary of calculation showing the sample spectrum, it's fitted curve and the residual curve of fitting. Values of pigment relations can be exported to text or excel files.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit am Leibniz-Institut für Agrartechnik Potsdam-Bornim e.V. selbstständig angefertigt habe und keine weiteren als die angegebenen Hilfsmittel und Quellen genutzt habe.
Ich versichere, dass die vorliegende Doktorarbeit an keiner anderen Hochschule als der Humboldt-Universität zu Berlin eingereicht wurde.

Michael Pflanz

Berlin, 29.08.2013